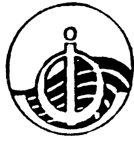

REGIONAL SEAS

UNITED NATIONS ENVIRONMENT PROGRAMME OCTOBER 1995

Quality Assurance and Good Laboratory Practice for marine microbiological measurements

Reference Methods For Marine Pollution Studies No. 66

Prepared in co-operation with

WHO

UNEP 1995

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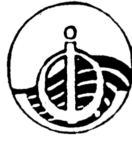

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PREFACE

The Regional Seas Programme was initiated by UNEP in 1974. Since then the Governing Council of UNEP has repeatedly endorsed a regional approach to the control of marine pollution and the management of marine and coastal resources, and has requested the development of regional action plans. The Regional Seas Programme at present includes 12 regions and has over 140 coastal states participating in it (1), (2).

One of the basic components of the action plans sponsored by UNEP in the framework of the Regional Seas Programme is the assessment of the state of the marine environment and of its resources, and of the sources and trends of the pollution, and the impact of pollution on human health, marine ecosystems and amenities. In order to assist those participating in this activity, and to ensure that the data obtained through this assessment can be compared on a world-wide basis and thus contribute to the Global Environment Monitoring System (GEMS) of UNEP, a set of Reference Methods and Guidelines for marine pollution studies is being developed as part of a programme of comprehensive technical support which includes the provision of expert advice, reference methods and materials, training and data quality assurance (3). The methods are recommended to be adopted by Governments participating in the Regional Seas Programme.

The methods and guidelines are prepared by, or in cooperation with, the relevant specialized bodies of the United Nations system as well as other organizations, and are tested by a number of experts competent in the field relevant to the methods described.

In the description of the methods and guidelines the style used by the International Organization for Standardization (IS0) is followed as closely as possible.

The methods and guidelines, as published in UNEP's series of Reference Methods for Marine Pollution Studies, are not considered as final. They are planned to be periodically revised taking into account the development of our understanding of the problems, of analytical instrumentation and the actual need of the users. In order to facilitate these revisions, the users are invited to convey their comments and suggestions to:

WHO/EURO Project Office
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48 Vassileos Konstantinou
P.O. Box 18019
GR-11610 Athens
GREECE

which is responsible for the development and preparation of microbiological and other health-related Reference Methods.

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(1) UNEP: Achievements and planned development of the UNEP's Regional Seas Programme and comparable programmes sponsored by other bodies. UNEP Regional Seas Reports and Studies No. 1, UNEP, 1982.

(2) P. HULM: A strategy for the Seas. The Regional Seas Programme: Past and Future, UNEP 1983.

(3) UNEP/IAEA/IOC: Reference Methods and Materials: A Programme for comprehensive support for regional and global marine pollution assessments. UNEP, 1990.

This first substantive issue of Reference Methods for Marine Pollution Studies No. 66 was prepared by the World Health Organization on the basis of a review of the Method during expert meetings and comments from individual scientists who tested the Method. The assistance of all those who contributed to this revised issue of the Reference Method is gratefully acknowledged.

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1. INTRODUCTION

The objective of a pollution monitoring programme is to obtain as accurate a picture as possible of the situation prevailing within any particular time-frame in order to determine whatever action is necessary on the basis of results obtained. In the case of microbiological monitoring of coastal recreational and shellfish areas, data obtained would be in the form of concentrations of specific microorganisms in defined volumes or weights of matrix (seawater, sand, sediment and shellfish). Such concentrations would normally be compared with prescribed standards to determine conformity with prescribed standards to determine conformity or otherwise, with eventual action being taken in line with that defined in the statutory instruments within which the relevant quality standards are incorporated. Where no standards exist, results would be expected to form the basis for decisions taken on health or other related grounds. Whatever the prevailing situation, monitoring data should determine whether any particular beach can be considered acceptable for bathing and/or other recreational usage, or whether any particular marine area is acceptable for shellfish growing or harvesting.

One of the essential requirements of any decision-making process is that the data on which a decision has to be based is accurate. The achievement of such accuracy is dependent on the means taken by any particular laboratory to assure the quality of data it produces. In programmes involving more than one laboratory, which is normally the case in national and international monitoring programmes, harmonization between the different laboratories, both in methodology and in quality assurance, is essential to ensure both reliability and inter-comparison of results obtained.

This part of the guidelines, which deals with laboratory quality control in microbiological monitoring of coastal recreational and shellfish areas, have been prepared by the World Health Organization within the framework of the Long-term Programme of Pollution Monitoring and Research in the Mediterranean Sea primarily for national laboratories participating in the microbiological component of this programme, though they are, broadly speaking, equally applicable elsewhere. They have been based primarily on the Handbook for Evaluating Water Bacteriological Laboratories (Geldreich, 1975), and the Guidelines for Health-related Monitoring of Coastal Water Quality (WHO/UNEP, 1977), and are intended to contribute both to the further improvement of monitoring activities in individual laboratories and to the harmonization and comparability of results obtained.

The original version of this part of the guidelines was reviewed by a WHO/UNEP Consultation on microbiological pollution of the Mediterranean Sea, held in Malta in December 1989. The present version incorporates the comments and suggestions made by participants at this meeting, as well as by a number of other microbiologists in the Mediterranean and other regions.

2. OBJECTIVES

The main objective of this part of the Guidelines is to provide useful advice for developing and implementing a quality assurance programme for laboratories engaged in the microbiological analysis of seawater and shellfish, or for improving such assurance programmes if already in operation. The ultimate goal is to ensure that

microbiological data collected have the greatest possible sensitivity, that microbiological results produced are reliable and comparable, and that interpretation and evaluation of such results are consistent with appropriate environmental quality criteria and standards for coastal bathing and shellfish-growing waters.

Specific objectives include the following:

1. To define the scope and contents of a quality assurance programme.

2. To identify the main aspects to be considered when collecting and preserving a representative water sample.

3. To describe the basic operating characteristics and quality requirements of laboratory equipment.

4. To discuss basic operating requirements for preparing and using laboratory utensils.

5. To examine proper sterilization methods for glassware, culture media, laboratory equipment and dilution water.

6. To summarize culture media specifications to be considered when making routine bacterial cultures.

7. To review the basic operating procedures of the multiple test tube method and the membrane filtration method for enumeration of total coliforms, faecal coliforms and faecal streptococci.

8. To examine practical methods for interpreting bacteriological results from coastal marine waters.

9. To describe practical methods for internal quality control and external quality control of bacteriological laboratories involved in monitoring coastal marine waters.

3. SCOPE AND CONTENT OF A QUALITY ASSURANCE PROGRAMME

3.1 Introduction

Various definitions of quality assurance and quality control are used in the literature. Although both terms are frequently used interchangeably, the definitions considered here are those proposed by Garfield (1984):

- Quality control is a planned system of activities whose purpose is to provide a quality product.

- Quality assurance is a planned system of activities whose purpose is to provide assurance that the quality control programme is actually effective.

As examples, the Reference Methods for marine pollution studies prepared by various UN Agencies and issued by UNEP's Regional Seas Programme, now re-issued in revised form in Parts II and III of these Guidelines, are components of a quality control programme, while the Guidelines for Health Related Monitoring of Coastal Water Quality (WHO/UNEP, 1977) and, more specifically, the series of intercalibration exercises on microbiological methods organized by WHO since 1982 are components of a quality assurance programme.

Studies and evaluations conducted in the Mediterranean region over the last decade have emphasized the need to ensure that quality control procedures are systematically and correctly applied by all participating laboratories. There is an obvious need for establishing and implementing a quality assurance programme capable of ensuring that existing quality control procedures and those that may be developed in the future are correctly applied by all microbiological laboratories in the Mediterranean. There is a need for a systematic evaluation of laboratory performance, both at the individual laboratory level and at the regional level, to detect deviations from the proposed quality control procedures and to assist in implementing correctly those procedures.

The following discussion on the scope and contents of a quality assurance programme have been based on two main references: the Handbook for Evaluating Water Bacteriological Laboratories (Geldreich, 1977) and Quality Assurance Principles for Analytical Laboratories (Garfield, 1984).

3.2 Quality Assurance Programme

The main objective of a quality assurance programme in a microbiological laboratory is to ensure the production of analytical results of high quality through the use of analytical procedures that are accurate, reliable, and adequate for the intended purpose.

The success of a quality assurance programme depends on the commitment and cooperation of the laboratory management and personnel, as well as on the adequacy of the objectives and foundation of the programme itself. Although the implementation of a quality assurance programme may initially represent a slight decline in productivity, and certainly requires human resources and time, the confidence and overall benefits derived from knowing that the results are reliable and comparable brings about higher personal satisfaction, improves laboratory performance and efficiency, and greatly contributes to the overall success of the monitoring programme, resulting thus in a worthwhile effort.

The development and implementation of a quality assurance programme requires basically: 1) a set of written guidelines and analytical procedures, 2) an organizational structure to carry out the quality assurance programme, and 3) a group of qualified and well trained laboratory personnel.

The following sections describe the basic elements of a quality assurance programme.

3.3 Quality Assurance Plan

A quality assurance plan is a prerequisite for a successful quality assurance programme. The three essential components of a quality assurance plan are: 1) a set of preventive measures, 2) a set of methods for the assessment, appraisal or evaluation of operating procedures, and 3) a set of remedial measures.

The preventive measures component includes a coordinated programme of planning actions and activities to be conducted before or during the analytical procedures to ensure that analytical systems are operating properly. Quality control planning, training of laboratory personnel, calibration of instruments, equipment maintenance, and routine standardization are examples of corrective measures to be undertaking in a microbiological laboratory.

The assessment, appraisal and evaluation component includes the systematic application of control measures to determine the accuracy and precision of the analytical determinations. The analysis of duplicate samples, the use of control charts, and the participation in intercalibration exercises are examples of the assessment component in an analytical laboratory.

The remedial measures component includes all the actions taken to determine the causes of inadequate accuracy or precision of analytical determinations, as well as the steps taken to restore proper functioning of the analytical system. The modifications introduced during the MED POL Programme on the culture medium and the incubation temperature specified in the method for determination of faecal streptococci are examples of corrective measures taken to improve the accuracy and specificity of this analytical method in the coastal waters of the Mediterranean.

Quality assurance may require from 10 to 20% of an analyst's time, with a substantial part of that time devoted to preventive maintenance and the analysis of check and reference samples. However, the benefits from a quality assurance programme are improved credibility of the laboratory, improved expertise of personnel, and improved staff morale. In summary, a more rational use of manpower and economic resources, and a more successful implementation of the monitoring programme for which the analytical determinations are conducted.

3.4 Quality Assurance Model

A quality assurance programme includes three basic levels of responsibility within the laboratory: 1) the scientific personnel, 2) the management personnel, and 3) the quality assurance unit. When the quality assurance programme covers a group of laboratories that participate in a common monitoring programme, as the microbiological monitoring component of the MED POL Programme, a fourth level of responsibility can be considered, in the form of a multi-laboratory quality assurance unit.

As Freeberg (1980) states, the first level of quality control rests on the trained bench scientist, who is responsible for doing the work properly, documenting it, and obtaining peer critique and review to assure that the work done meets relevant scientific standards. Unless competent and well-trained scientists carry out the microbiological determinations, there is no quality assurance programme or evaluation process that can produce quality results.

Laboratory management plays an important role in the quality assurance programme by providing adequate interaction with scientists, stressing the need for reliability and comparability of results, and providing adequate facilities, equipments and resources to allow scientists to work efficiently and adequately.

The responsibility of the quality assurance unit is to examine the adequacy of the facilities and resources made available to scientists, to evaluate their analytical procedures, and to modify, in coordination with scientists themselves, their analytical procedures in accordance with the results of the quality assurance programme.

In cases such as the MED POL Programme, in which laboratories from different countries participate simultaneously in a long-term monitoring programme, it is necessary to establish a higher level of responsibility for quality assurance. The main responsibility of this inter-laboratory unit is to ensure that quality assurance personnel from each participating laboratory are properly trained and work in close collaboration with each other.

It is extremely important that the responsibility for quality assurance be considered a positive contribution to improved performance and efficiency within the laboratories, rather than a policing effort on everyday activities.

3.5 Quality Assurance Objectives

A quality assurance programme must have a set of clearly defined objectives. Although the number and extent of those objectives vary according to the type of laboratory, the following are some of the objectives commonly considered in quality assurance programmes (USCPSC, 1979):

1. To maintain a continuous assessment of the accuracy and precision of data produced by laboratory analysts.

2. To provide a measure of the accuracy and precision of the analytical methods used in the laboratory, and to identify weak methodologies.

3. To establish training needs within the analytical group.

4. To keep a permanent record of instruments performance as a basis for validating data and projecting the needs for equipment repair and replacement.

5. To advance overall quality of laboratory performance.

6. To improve data recording and transcription, and foster preparation of writing reports on the progress of the quality assurance programme.

A clear understanding of the objectives of the quality assurance programme by all the personnel involved will result in a more efficient implementation of the programme.

3.6 Quality Assurance Programme Elements

A successful quality assurance programme requires a clear identification of its elements. In general, the elements of a programme cover all the activities carried out by the laboratory. Although some elements of the laboratory, such as facilities design, may not have a direct relationship with quality assurance, they all contribute to a more efficient operation and, ultimately, to a successful achievement of the quality assurance programme.

Among the elements that may be included in a quality assurance programme the following can be considered (Ratliff, 1980; NIOSH, 1976):

1. Definition of the objectives.
2. Planning for quality assurance.
3. Training on quality assurance.
4. Identification and control of samples.
5. Handling, storage and delivery of samples.
6. Preventive maintenance and calibration of equipment.
7. Standard operating procedures.
8. Reagent and reference standards.
9. Statistical quality control.
10. Data validation and interpretation.
11. Laboratory analysis and control.
12. Intra-laboratory and inter-laboratory calibration.

The number and extent of the elements actually included in a quality assurance programme will vary depending on the type, size, and quality needs of the laboratory concerned.

3.7 Quality Assurance Coordination

The implementation of a quality assurance programme should be the direct responsibility of the person designated as the quality assurance coordinator. The coordinator should be preferably responsible to laboratory management and be located organizationally outside the laboratory. It is extremely important that the quality assurance coordinator maintains a high level of objectivity and a close collaboration with management and scientists.

Two basic responsibilities of the coordinator are:

1. To make recommendation on quality assurance policies and to assist in its formulation within the laboratory, as far as it concerns laboratory policies, administrative support, and training of laboratory personnel.

2. To provide quality assurance guidelines for data reduction and interpretation, maintenance and calibration of instruments, and planning of quality assurance evaluations, as well as to participate in those quality assurance evaluations.

In monitoring programmes with a considerable number of laboratories, it may be necessary to have a quality assurance group, whose main responsibility would be to evaluate the analytical methodologies and procedures used, and to assist scientists and technicians in implementing any necessary corrective measure. A quality assurance coordinator at the national level could be established to supervise and assist all the laboratories in each country.

3.8 Quality Control Protocol

Every laboratory operating under a quality assurance programme should have a quality assurance protocol or manual that specifies the standard operating procedures. A quality assurance protocol or manual could be defined as a written document that identifies the policies, organization, objectives, functional activities, and specific quality assurance activities designed to achieve the quality goals desired for the operation of the laboratory (USEPA, 1980).

The US Environmental Protection Agency (1976) outlines the more relevant components of a quality assurance protocol. Among them, the following deserve special attention:

1. Organization of the laboratory.

2. Quality assurance objectives. Statistical terms for quality control, such as accuracy and precision, should be defined.

3. Quality assurance system. The relevant elements of the quality assurance programme should be described.

4. Evaluations of performance. The frequency and methodology for evaluating the quality control of the laboratory should be specified.

5. Correctives measures. The actions to be taken to correct the observed deficiencies in quality control should be documented.

6. Reporting forms. All the reporting forms used for data recording and quality assurance evaluation should be included.

7. Quality assurance reporting. The format and frequency of the quality assurance reports should specified, together with the distribution list for those reports.

3.9 Practical Implementation

Before starting a quality assurance programme, or modifying an existing one, Garfield (1984) recommends to follow a series of steps that can be summarized as follows:

1. Prepare a quality assurance plan in close collaboration with all laboratory personnel, management and scientists.

2. Evaluate the cost involved in implementing the quality assurance programme, with particular attention to the time and effort that laboratory personnel are willing to devote to the programme.

3. Define the objectives of the quality assurance programme, taking into account the level of quality desired or required for the work performed.

4. Identify the quality assurance elements necessary, taking into account the discussions and recommendations included in the following chapters of these Guidelines.

5. Elaborate the quality assurance protocol that will be used as the reference document for the programme.

6. Designate a quality assurance coordinator or group, and specify his (their) responsibilities.

7. Establish the frequency and format of the quality assurance evaluation exercises.

8. Define the procedures for implementing future corrective actions.

9. Prepare an outline of the different types of evaluation reports, establish the person(s) responsible for their preparation, and specify the circulation list.

10. Implement the quality assurance programme, evaluate its progress and modify its contents based on need and experience.

11. Document all the activities in detail.

The following chapters cover the most relevant elements of a quality assurance programme for a microbiological laboratory. The discussions and recommendations included in those chapters can be adequately incorporated into a quality assurance protocol, the reference document for all quality assurance activities carried out in a microbiological laboratory.

4. SAMPLING

4.1 Introduction

Monitoring and surveillance programmes commonly rely on observations made on discrete samples obtained within spatial and temporal constraints. Ensuring that the sample obtained is representative of the phenomenon under study is thus an essential component of a monitoring programme. In contrast to what is commonly believed, it is very important to realize that errors introduced during the sample collection and preparation process are usually several orders of magnitude higher than errors due to analytical determinations.

The main difficulty in obtaining a representative water sample relates to the lack of homogeneity, both in space and in time, of the water mass under consideration. This difficulty is especially important when sampling natural waters, either inland or coastal waters, due to the unpredictable inputs of storm water runoff and of municipal and industrial effluents, whose quality will vary according to influent quality and performance of wastewater treatment plants. In addition, variable receiving water circulation patterns greatly influence dispersion and transport of discharges.

The main aspects to be considered for obtaining a representative water sample are; 1) the adequate selection of the sampling station, 2) the strict adherence to proper sampling procedures, 3) the complete identification of the sample, 4) the adequate preservation of the sample, and 5) the prompt transport of the sample to the laboratory.

4.2 Sampling Stations

Sampling stations for monitoring recreational waters should be selected as to reflect the water quality within the entire recreational area. There is no definite criterion for establishing the number or the spacial distribution of the sampling stations required for monitoring the microbiological quality of a recreational area. One commonly used criterion is to establish sampling stations in the areas of higher bather density, and especially in those points known to be affected by discharges from nearby storm drains, public rest rooms, recreational buildings, or outlets of submarine outfall structures.

Quantitative observations in Mediterranean coastal waters indicate that recreation takes place mostly in the zone ranging from the water line up to points of 2.0 m depth, where bathers can regularly stand upright. However, most children are located in areas close to the water line, a zone where breaking waters entrain considerable concentrations of sand and sediment particles. Sampling stations are thus commonly established at points where the water depth ranges from 1.0 to 1.5 m. Very limited experimental information is available on which to base the selection of sampling locations.

A baseline study of water quality in the recreational area provides an excellent basis for establishing the location and number of sampling stations. The results of this intensive sampling programme, together with a detailed survey of water currents and water discharges, will reveal any particular pattern of water quality deterioration that has to be considered when selecting sampling stations representative of the whole recreational area. Specifically, this study should include the analysis of water samples

taken at different water depths, at different hours of the day, during different tide phases, and during any other known source of possible water quality variation.

The experience gained during the implementation of the monitoring programme should serve to modify and improve the initial sampling programme. It is generally recommended that water samples be collected from the upper layer of the water mass, but always below the surface. The objective is to obtain a representative sample of the water mass to which most bathers are exposed while discarding any possible analytical interference due to particulate or floating material, or other localized phenomenon occurring at the air-water interphase. The Reference Methods for marine pollution studies prepared by UNEP, the EEC Directive 76/190 (1976) and other regional monitoring guidelines (Generalidad de Cataluña, 1983) all recommend that water samples should be obtained at 20-30 cm below the water surface. Very limited experimental information is available on which to base the selection of sampling depth.

Sampling frequency should be directly related to the intensity and temporal pattern of recreation activities. Sampling should preferably be conducted during times and days of higher bather density. Very limited experimental information is available on the diurnal or other temporal variation on water quality. This might be unique to the recreational area under study.

Furthermore, sampling frequencies established by the EEC Directive 76/160 (1976) and the interim environmental criteria for bathing water in the Mediterranean approved by The Contracting Parties to the Barcelona Convention (UNEP, 1985) specify only minimum requirements of one sample every fifteen days. Laboratory working schedules, particularly during the summer season, the peak bathing season in the Mediterranean, impose real limitations on the time of the day and the days of the week when samples can be collected, as they have to be brought to the laboratory and analyzed on the same day. This limitation also applies to sampling during summer week-ends and holidays, when recreation activities usually reach their highest annual intensity.

In summary, there is a clear need for sound selection criteria concerning the location, the frequency and the timing of water sampling. The general guidelines presented here represent mostly a compromise between conventional operation schedules of laboratories and the increasing needs for monitoring the recreational water quality. The systematic application of a set of agreed sampling procedures would certainly represent a first step in promoting the reliability and comparability of the results obtained by laboratories participating in the MED POL Programme. Specific studies should be conducted to establish more specific criteria relating to location, frequency, and timing of sampling, so more adequate protocols can be established in the future.

There is no criterion for determining the number of sampling stations to be established in a recreational area. However, experience gained after several years of monitoring activities may provide a sound basis for modifying the number and the location of the initial sampling stations. Nonetheless, it is extremely important that, once the sampling stations have been established, no changes be made until the bathing season is finished, and only when the proposed changes are adequately justified.

The frequency of sampling commonly adopted in monitoring programmes in the Mediterranean is one sample per week during the 12 weeks of the summer season, and

one sample every two weeks during the rest of the year. Higher sampling frequencies have been used in some coastal areas. The analytical capacity of the laboratories and the sampling frequency adopted will determine the total number of sampling stations that can be included in a monitoring programme. It is clearly preferable to maintain those sampling frequencies on a limited number of sampling stations than to cover a larger number of sampling stations, but using a sampling frequency so low as to render unfeasible any meaningful water quality evaluation.

The time of sampling most commonly used in the Mediterranean is the morning hours, as close as possible to the noon time period, when most bathing activities take usually place. The sampling time normally recommended is from 9 a.m. to 2 p.m., and preferably from 12 p.m. to 1 p.m., the limiting factor being generally the transport time to the laboratory and the time to analyze the sample during summer working hours.

4.3 Sample Collection and Size

Efforts should be made to collect samples that are representative of the water mass under consideration and to ensure that samples do not become contaminated at the time of collection or before they are analyzed. Sterile sample bottles for microbiological analysis must remain closed until the time of collecting the sample. The bottle should be closed immediately after sampling, adequately identified and placed in a protective container for conservation and transport to the laboratory.

Bacteria adsorb to particulate material and to the inner walls of sampling bottles. Therefore, an adequate air space must be left in the sample bottle when obtaining the sample to allow subsequent mixing before analysis. Extreme differences in microbial concentrations became apparent during one of the intercalibration exercises held in the Mediterranean (UNEP/WHO, 1986) due to inadequate sample mixing, after heavy rainfall resulted in an increase of suspended material in the coastal waters under study. Under no circumstance should the analyst discard a portion of the sample to allow for better mixing of the bottle contents. Bottles with no air space should be thoroughly and carefully mixed before analysis, by repeated rapid inversions of the closed container prior to opening.

The minimum sample size collected for analysis should be 100 ml for each bacterial indicator to be analyzed. When the three common bacterial indicators are analyzed it is recommended to obtain a 500 ml sample volume. Microbiological analysis requiring sample concentration, such as virus determination, may require sample volumes of 50 litres or more.

4.4 Sample Collection Procedures

The basic procedure for collecting a sample is to hold the bottle near its base, to introduce it below the surface of the water, and to remove its cap so it can be filled with water at the desired water depth. As the bottle fills, it should be pushed gently forwards through the water to prevent contamination from the sampler's hand. When sampling by means of a rope or an extension arm, the bottle can be introduced open and neck downward below the water surface, and then be turned up until the neck points slightly upward. When sampling from a boat, samples should be collected from the upstream side of the boat to prevent any contamination from the sampling boat.

When using bottles with ground glass stoppers, a piece of paper is usually inserted between the bottle mouth and the stopper to facilitate opening after sterilization. This piece of paper should be removed before reinserting the glass stopper to prevent the risk of sample contamination or sample spilling from an inadequately closed bottle.

All samples obtained from a water mass suspected of having some residual chlorine must be dechlorinated at the time of collection. Before sterilizing sampling bottles, a sufficient amount of sodium thiosulfate should be added to each bottle so that after the water sample is collected, the concentration of dechlorinating agent is close to 100 mg/l. A 10% sodium thiosulfate solution is commonly used for dosing the sampling bottles. The addition of 2 drops of this solution for each 100-ml of capacity of the bottle provides an adequate concentration of dechlorinating agent in the bottle.

Bottles should be clearly identified with a number permanently marked in the bottle or written with a water proof pen on the bottle walls. It is required that the sample collector fills out the sample identification form immediately after taking each sample. Samples received in the laboratory with inadequate report forms or questionable identification should be discarded.

In monitoring programmes, water sampling is commonly only one element of the whole data collection effort, in which weather conditions, water transparency and temperature, and other environmental conditions are usually measured and recorded. The sample collector should write adequate remarks concerning water quality conditions at the time of sampling that may suggest unusual levels of bacterial contamination. This information will be very helpful to microbiologists when preparing adequate dilutions for analysis.

Laboratory personnel must be responsible for the reception, custody, care and processing of the sample upon arrival at the laboratory. They should maintain a logbook to show registration of the sample upon reception from the sample collector, as well as indication of the date, arrival time, and the signature of the sample collector.

4.5 Sample Conservation

All water samples must be examined as soon as possible after collection. Conservation time is critical for monitoring recreational water quality. Sampling stations are usually far away from laboratory facilities. Traffic congestion during the summer bathing season and beach accessibility may further extend the time required for sample transport to the laboratory. It is usually recommended that sample conservation time should not exceed 6 hours after collection. The samples should be kept protected from exposure to sunlight and heat. They should be preferably kept in picnic coolers, where cold chemical packs can be introduced to maintain the temperature close to 4 °C during transport. Samples should be processed within 2 hours upon reception in the laboratory to ensure the quality and validity of the results. The delayed-incubation test (Standard Methods, APHA, 1985) may be used when it is impractical to apply conventional procedures.

Studies conducted on the evolution of faecal indicator concentrations as a function of conservation time (Mujeriego, 1985) indicate that the decrease in bacterial concentrations in coastal water samples, after being kept for 26 hours at 4 °C, are within the significance level of the bacteriological methods of analysis. Consequently, there

was no statistically significant difference between the bacterial concentration of a sample analyzed immediately after collection and that obtained when analyzed 24 hours after collection. On the other hand, deviations of several orders of magnitude were observed due to inadequate application of bacteriological methods by the participating laboratories.

When sample transit time does not allow the use of a central laboratory, other alternatives must be considered, such as: 1) analysis of samples in an approved laboratory located nearby, 2) examination of the samples with an approved laboratory field kit, and 3) on-site analysis using a mobile laboratory. Examination of sea water with an approved laboratory field kit or a mobile laboratory should undergo a thorough testing and comparison with an approved laboratory before they are adopted as an acceptable procedure.

When a water sample is lost or discarded for subsequent analysis, an effort should be made to obtain an additional sample on the following days. Although it is not possible to obtain a replicate recreational water sample, it is very convenient to maintain the number of samples as close as possible to that initially scheduled. An inadequate number of experimental results may complicate considerably the subsequent evaluation process, especially when calculating the statistical precision and significance of the experimental results.

4.6 Summary

In summary, water sampling is an essential element of a quality assurance programme. Once the detailed procedures for collecting and preserving a representative water sample are established, it is essential that they are systematically applied by all participating laboratories. Deviations in bacteriological concentrations caused by disregard to systematic sampling procedures are usually much higher than those produced by microbiological methods of analysis. Although there is still need for sound criteria for selecting sampling station location, sampling time, sampling frequency, and to a lesser extent for sample conservation, the systematic application of currently recommended procedures ensures the comparability of data obtained by different laboratories. The experience gained and the studies conducted during the implementation of the MED POL Programme should greatly contribute to further advance the development of water sampling criteria.

Proper selection, training and consideration of sampling staff are essential elements of any monitoring programme of coastal marine waters. Provision for adequate means of transportation, and a well defined schedule for sample collection should greatly contribute to the successful implementation of the monitoring programme. A monitoring programme should not be established when sample collection represent an additional task to persons with multiple other responsibilities, or in the absence of a practical training programme covering all the aspects discussed above.

5. LABORATORY EQUIPMENT

Laboratory equipment must be of adequate quality to achieve the levels of sensitivity and reliability required by microbiological methods. Furthermore, it should be designed and built as to require minimum service repairs to correct any deviation o

failure of its essential characteristics. It is recommended that user and reference manuals be read by all technical personnel for proper understanding and operation of each piece of equipment. The manuals should be available in the laboratory files for reference when the equipment has to be repaired or one part has to be replaced. To prevent undue accidents, all laboratory personnel must have a thorough understanding of the operational controls and adequate use of the different pieces of equipment. All laboratory equipment should be subject to a stated policy of service and calibration to check its adequate operational performance.

5.1 Air Incubators

Incubation temperature is an essential selection parameter for the analysis of microbial indicators in bathing waters. Each analytical method has an incubation temperature requirement, obtained after extensive comparative studies. The recommended temperature has been selected as the optimum for the detection and recovery of the microorganism of interest. Incubation temperature, together with the length of the incubation time and the basic culture medium, serve to define one type of microorganism.

Metabolic activity of microorganisms is directly related to incubation temperature. In particular, incubation temperature has a direct influence on the rate of gas production, a basic characteristic used in the detection of coliform organisms. Studies conducted on agar plate counts for *Escherichia coli* (Taguchi, 1960) indicate that 35 °C was the optimum incubation temperature. The next best temperature was 37 °C. Incubation temperatures below 35 °C increase the possibilities of interferences caused by false positive colonies of noncoliform organisms. For this reason, incubator temperature tolerance must be accurately measured within ± 0.5 °C, and all thermometers used in these analyses must include 0.5 °C scale divisions.

In the absence of recording thermometers, incubator temperature should be recorded daily, preferably in the morning and in the afternoon. Incubation temperature deviations greater than 0.5 °C from the 35 °C required temperature should be corrected by thermostat adjustment.

To ensure a uniform temperature inside the incubator, culture tubes and plates should be arranged as to prevent overcrowding and to ensure adequate air circulation among microbial cultures. Overcrowding frequently results in hot and cold spots within the oven that interfere with the results of the analyses. Petri dishes should not be incubated in stacks of more than 5 plates, and ample space should be left between the stacks on the shelves to ensure an adequate air circulation within the oven.

Air incubators operated at 35 °C create a low-humidity environment that may affect broth and agar cultures kept during long incubation periods. Agar plates incubated 48 hours at 35 °C should not experience more than a 15% weight reduction through desiccation. Water losses through evaporation cause unfavorable pH changes in broth cultures, and can suppress bacterial growth or reduce the size of the colonies grown in membrane filters. Some commercial incubators have a built-in water reservoir to help maintaining the humidity at approximately 75 to 85%. These reservoirs must be periodically filled with water to replenish water lost through evaporation. A beaker filled with distilled water can also help maintaining the desired relative humidity in the incubator chamber.

5.2 Elevated Temperature

The analytical methods recommended for the selective recovery of faecal coliforms require an incubation temperature of 44.5 °C. Incubation temperature must be precisely controlled since temperatures lower than those recommended will allow the growth of nonspecific organisms, while higher temperatures, will reduce the recovery of faecal coliforms. It is recommended that incubation at this high temperature be conducted using a water bath incubator, because this system allows a more precise temperature control than air incubators. However, modern air incubators with electronic temperature control can maintain a temperature variation of ± 0.2 °C. Accurate temperature measurement and control are essential for elevated temperature tests. In the absence of a recording thermometer, water bath incubator temperature should be recorded daily using an immersed thermometer or digital electronic thermometer.

Since water bath temperature tolerance must be accurately measured within ± 0.2 °C of 44.5 °C, all thermometers used for this purpose should include 0.1 °C scale divisions as a requirement.

Large bench-top water baths with covers can effectively maintain a temperature of 44.5 °C within ± 0.5 °C. Some non circulating water baths are capable of temperature control within ± 0.2 °C, while others may exhibit a slightly greater deviation. These latter water baths can be brought to within ± 0.2 °C temperature tolerances by installing a low speed stirring motor or an external water pump to create a gentle circulation of water and thus prevent any thermal stratification. To avoid problems of metal corrosion, stainless-steel or plastic-coated baskets or racks should be used in water baths. Water bath incubators can be cleaned and disinfected using liquid laundry bleach at a rate of 1 ml per 10 liters of water. After a 24-hour contact period, the bath should be drained, and then flushed and refilled with distilled water.

5.3 Dry Heat Sterilization

Dry heat sterilization of laboratory items is achieved by maintaining the temperature at 170 ± 10 °C during 2 hours. Commercial-type ovens should be checked to verify they satisfy those requirements. It is recommended that any sterilization oven used in the laboratory be provided with an accurately calibrated thermometer. In the absence of a recording thermometer, the oven should be provided with a long-stem thermometer in the range of 160-180 °C, inserted through the ceiling port and with the bulb introduced in a graduate cylinder filled with sand. Temperature should be verified during the sterilization process. Commercially available temperature indicators are useful for sterilization control.

Items to be sterilized should be placed conveniently separated from each other to allow air circulation and to ensure that all items reach the sterilization temperature.

5.4 Autoclaves

Wet sterilization of numerous laboratory items is achieved by maintaining a temperature of 121 °C during 15 minutes, using an autoclave. Bacteriological media, sample bottles, membrane filter equipment, and test culture discards are sterilized in an autoclave. The autoclave should be equipped with an accurate thermometer with its bulb properly located in the exhaust line so that it registers the minimum temperature

in the sterilization chamber. The sterilization period should be counted from the time when the required chamber temperature is achieved.

Household pressure cookers may be used in emergency service if equipped with pressure gauges and thermometers with bulbs located 3 cm above the water level. However, they are not considered the equivalent of an autoclave recommended for permanent laboratory facilities, mainly because they are difficult to regulate.

Labelling tapes with heat-sensitive colour changing inks, heat sensitive crayons, or other materials that change colour or physical state when sterilization temperature is reached, are useful for autoclave temperature control. These sterilization indicators should be used each time the autoclave is operated. Autoclaves must receive periodic inspection and preventive maintenance to ensure proper operation and prevent accidents from occurring. Periodic cleaning of the interior surfaces, systematic inspection and cleaning of drains, and replacement of old rubber gaskets and temperature and pressure gages are essential components of a preventive control programme.

5.5 Thermometers

To ensure that all thermometers routinely used to monitor temperatures in the laboratory are accurate, their readings should be verified by comparing them with those of certified thermometers. Considering the importance of exact temperature control, each laboratory should have a set of certified thermometers.

Since thermometers accuracy is not uniform over their entire temperature range, they must be verified for accuracy within the minimum and maximum range of intended used. Thermometers should be checked periodically for hairline breaks in their mercury columns as they decrease measuring accuracy.

5.6 pH Meters

pH measurements in a microbiological laboratory must be made with an electronic pH meter capable of reading ± 0.1 pH units. As electrodes may become defective and cause erratic readings, it is recommended that a spare replacement electrode should always be available. pH meters must be periodically calibrated using buffered solutions of known pH value. Since calibration is not uniform over the entire pH range, calibration should be conducted every time the pH values to be read are outside the range for which the equipment was previously calibrated.

Colorimetric pH measurements are not acceptable in the bacteriological laboratory, because it is impossible to make a colorimetric pH determination of strongly coloured solutions such as M-Endo broth or M-FC broth used in the membrane filter method.

5.7 Balance

Preparation of culture media may require a balance capable of weighing several hundreds grams. For this purpose, each laboratory should have a balance with a sensitivity better than 2 grams at a 100 grams load.

Weighing media additives, reagents, and dyes, which are added in amounts smaller than 2 grams, requires an analytical balance with a 1 mg sensitivity at a 10 grams load. This type of sensitive balance must be protected from vibrations, dust and disturbances created by air conditioning systems or laboratory traffic. Special attention should be paid during weighing procedures to protect the delicate knife edge on the balance point.

5.8 Light Source

A 10x to 15x magnification is the best for counting MF colonies. Direct visual examination of MF total coliforms cultures is not recommended because small colonies or those with atypical colour may not be detected.

The diffuse daylight from a cold-white fluorescent lamp, adjusted to an angle of 60 to 80 degrees above the MF culture, is the most adequate for observing the golden metallic sheen of coliform colonies, the blue colour of faecal coliforms colonies, or the red colour of faecal streptococci colonies.

5.9 Inoculating Tools

Several types of inoculating tools are commonly used in the bacteriological laboratory to transfer bacterial growth from one culture medium to another or to a microscope slide. Among those most commonly used are the single wire loop, the disposable hardwood applicator stick and the Pasteur pipette.

The single loop should have a diameter of 4 mm or greater, preferably between 6 and 7 mm, to allow adequate transfer of culture broth without accidental spillage. The wire shank of transfer loops should be from 7 to 8 cm long, to allow reaching the culture broth without contaminating the tube with the loop holder. Alternatives to the wire loop for culture transfer are the single-service transfer loops of aluminum or stainless-steel. These transfer loops may be placed in a metal-foil cover and sterilized either by dry heat or in an autoclave. Sterile disposable plastic transfer loops can also be used.

Disposable hardwood applicator sticks, sterilized by dry heat, may also be used for transferring broth cultures. Autoclave sterilization must not be used because the wood distillate products that may be generated are toxic to bacteria during the transfer procedure.

Pasteur pipettes are not recommended for transferring culture broth because of the excessive quantity of inoculum that may be introduced in some cases. The large number of various organisms introduced in this way may not be adequately suppressed by the selective substances of the culture medium, resulting in a considerable number of false positive metabolic reactions.

5.10 Membrane Filtration Assemblies

Filtration assemblies used in MF methods have two parts: the funnel and the funnel receptacle. Reusable filtration assemblies may be constructed of autoclavable plastic, borosilicate glass, stainless steel, or metal plate. Funnels manufactured of

stainless steel are more resistant to corrosion and are very durable under field use. Glass and plastic funnels cost less, but they are subjected to accidental breakage.

The funnel portions of each filtration assembly must be washed at least once per week in a mild detergent solution to prevent the accumulation of dirt or water hardness spots on the funnel walls.

5.11 Forceps

Membrane filter methods require the use of sterile forceps, both for placing the sterile membranes on the funnel receptacle and to transfer the membrane to the culture medium. Forceps must be sterilized by flaming them with alcohol. Forceps should have smooth ends, similar to those used in stamp collection, to prevent puncturing or tearing of the membranes. A metal file can be used to smoothly round the inner surfaces of sharply pointed forceps.

6. LABORATORY UTENSILS

Laboratory glassware utensils are subjected to a variety of corrosive substances during testing procedures, high temperature during sterilization, repeated cleaning procedures, and continuous handling; all of these aggressions speed glassware to ultimate discard and replacement. An extensive choice of plastic and disposable hard-glass (borosilicate) items, and some stainless-steel vessels are currently available as an alternative to glassware. Plastic materials used in a bacteriological laboratory must be free from toxic residuals used in the molding process, have accurate calibration marks, and withstand repeated autoclaving if they are to be reused.

6.1 Media Preparation Utensils

It is recommended that utensils used for preparing culture media be made of borosilicate glass or other suitable noncorrosive material, such as stainless-steel. Utensils made of aluminum, copper, or zinc alloys should not be used because these metals also react with media solutions and introduce metal ions that are toxic to bacteria. Furthermore, utensils for media preparation must be thoroughly cleaned to prevent cross contamination with residues or dried medium.

The following sections summarize the most important recommendations to be taken into account when preparing and using sampling bottles, pipettes, Petri dishes, culture tubes, dilution tubes and bottles, membrane filters, and absorbent pads.

6.2 Sampling Bottles

Sampling should be conducted using wide-mouth sample bottles because they facilitate and speed sample collection. Glass sample bottles should be made of borosilicate or other noncorrosive glass, preferably with metal or plastic screw-cap closures. New plastic screw caps should be checked for bacterial toxicity by an adaptation of the pure water test described in Standard Methods (APHA, 1985). Plastic caps can be generally detoxified of phenol residuals by six successive autoclavings with repeated changes of distilled water.

Before sterilization is conducted, ground-glass stopper closures should be covered with a metal foil or heavy impermeable paper that extends from the cap to the shoulder area of the bottle. It is preferable to use foil because they can be held in place by pressing the foil around the bottle neck. This cover should be kept always over the ground-glass cap while handling; the cap and its cover should be replaced and pressed over the bottle once the sample is collected.

Plastic sample bottles and their screw closures must be of the same autoclavable plastic material to prevent deformations during autoclaving and subsequent leakage. Screw caps of sampling bottles should be loosely closed during autoclaving to facilitate changes in air pressure and prevent collapse and deformations of the bottles. The main advantages of plastic bottles for bacteriological sampling are their low cost, their light weight, and their resistance to breakage. However, they must be free of toxic substances or organic matter introduced during their manufacturing process.

6.3 Pipettes

Pipettes use in the bacteriological laboratory must deliver the specified volume quickly and accurately within a 2.5% tolerance. Glass and disposable plastic pipettes must be sterile, meet the required accuracy and legibility, and be free of toxic residues. To prevent the technician from accidentally ingesting any dangerous substances or microorganisms present in water samples, it is recommended to use a hand pump or to insert a cotton plug into the mouth end of the pipettes.

Sterilization and storage of sterile pipettes are conveniently done using metal boxes or cans. These containers should be of stainless-steel because they resist heat deterioration without introducing any toxic substances to bacteria. Furthermore, metals boxes or cans allows simple access to the pipettes while providing convenient storage for large number of pipettes. Sterilization and storage of sterile individual pipettes may be achieved by wrapping them in good quality paper that resists charring caused by sterilization temperatures.

6.4 Petri Dishes

Petri dishes are essential laboratory utensils for standard plate count determinations, MF cultures, and streak plate isolation of bacterial cultures. The size of Petri dishes commonly used for pour plates and pure culture isolation is 100 mm x 15 mm. Since the MF methods use standard 47-mm diameter membranes, tight fitting 50 mm x 12 mm Petri dish are generally used for this examination method. However, loose fitting Petri dishes can be used, provided they are kept in closed containers during incubation in water baths; larger size Petri dishes can also be used, where several membranes can be properly placed.

Disposable Petri dishes have considerably replaced glass Petri dishes traditionally used in the microbiological laboratory, because of their lower cost, the simplification of washing and sterilization procedures, and their lower risk of brakeage. Plastic dishes with tight fittings covers are preferred to the standard Petri dishes with loose-fitting covers for the MF cultures, because they reduce evaporation losses from broth or agar media, and they maintain a humid atmosphere in the culture dish.

Sterilizing and storing of glass Petri dishes should be done using metal containers (stainless-steel or aluminum), because they ensure that the loosing tops remain together, therefore preventing dust contamination of the sterile inner parts of the dish. When metal containers for sterilizing the 60-mm-size glass-type or the 100-mm-size plastic-type Petri dishes are not available, heavy metal foil or strong wrapping paper can be used to wrap approximately 6 to 10 dishes for sterilization and subsequent storage.

Disposable plastic Petri dishes are generally sold presterilized and packaged in plastic bags as a protection against contamination. Tight fitting disposable Petri dishes for MF cultures are package in small cardboard boxes, which are also useful as storage containers during the first use of the dishes and during subsequent reuse after sterilization.

6.5 Culture Tubes and Closures

Culture tubes are essential laboratory utensils for the multiple test procedures, biochemical tests for bacterial identification, and stock culture collections. Culture tubes must be made of borosilicate glass or other corrosive resistant glass. Disposable culture tubes are generally made of soft glass and therefore are not recommended for bacteriological use because of reaction between the glass and culture media during storage and incubation.

The size of culture tubes must be sufficient to contain the culture medium and the sample volumes employed, leaving enough free space. The size of the inverted vial used in fermentation tubes should be related to the culture and medium volumes. Large fermentation vials make early observation of gas bubbles more reliable.

Snug-fitting stainless-steel or plastic caps, and loose fitting aluminum or lignin caps are the recommended closures for culture tubes used in the multiple tube method. Although nonabsorbent cotton plugs may be used also as tube closures, their adequate preparation requires considerable time.

6.6 Dilution Tubes and Bottles

Bacteriological examination of coastal water samples by either the multiple tube method, the membrane filter method, or the pour plate technique generally requires preparation of accurate sample dilutions. Dilution water blanks may be prepared in either screw-cap culture tubes containing 9 ml of diluent for 1:10 dilutions, or in dilutions bottles with capacity for 99 ml diluent. Dilution bottles are commonly used since they allow preparation of both 1:10 and 1:100 dilutions.

Glass dilution bottles must be made of borosilicate or other corrosion resistant glass; it is convenient that a graduation level for 9 ml (tubes) or 99 ml (bottles) be permanently marked on the glass wall. Although this mark aids the bacteriologist in preparing the dilution blanks, it is also possible to carefully measure the volume of dilution water required to obtain the 9 ml or 99 ml of dilution water after sterilization. It is recommended that the especial dilution tubes and bottles should only be used to prepare dilution water blanks.

Closures for dilution tubes and bottles must prevent leakage of the contents during vigorous shaking or mixing to obtain uniform bacterial suspensions. Therefore,

the recommended closures are ground-glass stoppers, rubber stoppers, or plastic screw caps. As mentioned previously, new plastic screw-cap closures should be checked for bacterial toxicity, and detoxified accordingly.

6.7 Membrane Filters

Analytical performance of membranes filters may vary as a result of manufacturing technology, materials used, and degree of quality control employed. Membrane filters for bacteriological examinations must ensure a complete retention of organisms on the surface of a nontoxic, inert matrix, and allow a continuous contact of those microorganisms with the nutrients present in the culture medium placed below the membrane. These basic conditions place demanding requirements on the quality of commercial brands of membrane filters used in the laboratory. Studies conducted by Tobin and others (1980) showed the relative performance of different brands of membrane filters.

Some inks used to imprint the grid system on the MF have been found to be bacteriostatic or bactericidal. It is therefore recommended to check the MF used for possible localized effects on bacterial growth.

Culture media for the membrane filter method have been developed to ensure adequate diffusion through the nitrocellulose matrix, while preventing any reaction with the chemical substances included in the culture media. These circumstances should be taken into account when selecting new types of membrane filters. The membrane filters selected should remain inert material to bacterial action and should not affect the physico-chemical characteristics responsible for media selectivity and specificity.

Membrane filters may be reused several times, provided they are used only in the same medium cultivations. However, there is no solid data on the performance of the reused filters. To reuse membrane filters, discarded filters are washed in three successive changes of gently boiling water, then boiled in 3 percent hydrochloric acid for 3 minutes. The filters are then washed in at least 3 changes of gently boiling distilled water. A trace of bromocresol purple indicator and sufficient sodium bicarbonate to neutralize any residual acidity are added to the final rinse water. Following a 5-min boil in this final rinse water, the filters are ready for reuse (Geldreich, 1975). The pink colour produced by the M-Endo type medium may be removed by presoaking the membrane filters in a 10% sodium sulfite solution before proceeding to the acid and neutralizing procedure.

6.8 Absorbent Pads

Bacteria retained on the membrane filter may receive nutrients from a either a broth-saturated absorbent pads or an agar-based medium. When the liquid medium is preferred, the absorbent pad substrate must be of high quality paper fibers, uniformly absorbent, and free of substances that could inhibit bacterial growth. Toxic materials present in absorbent pads may be removed by soaking the pads in distilled water held at 121 °C for 15 minutes in an autoclave, followed by sterilization of the absorbent pads at 121 °C for 15 minutes.

An alternative approach to absorbent pads is to prepare all MF broths on an agar-based form, by adding 1.5 percent agar or the agar proportion recommended by

the manufacturer. Agar preparations may be used immediately or stored in a cool, dark place and used any time within the storage time recommended for each culture medium, provided no dehydration occurs.

7. CLEANING AND STERILIZATION

Thorough cleaning of glassware and proper sterilization of media and equipment are essential elements of a properly functioning laboratory. Without careful attention to these services, the sensitivity and reproducibility of bacteriological examinations will be affected, impairing the quality of laboratory results.

7.1 Cleaning of Glassware

Laboratory glassware can be cleaned using mechanical washing equipment or by hand washing methods. Automatic washing equipment should be able to obtain sparkling clean glassware, free from acidity, alkalinity, and toxic residues that could suppress the growth of microorganisms. When hand washing is employed, detergent formulas for laboratory use should be employed rather than the mild compounds commonly used in home dish washing. Hot wash water must be used and items should be carefully brushed to ensure removal of normal film deposits and residual deposits of dried material. After washing is completed, glassware must be rinsed, first with hot water and then with distilled water, to ensure complete removal of the washing detergent and any chemical deposits. Glassware items must be inspected after air drying for sparkling clarity.

7.2 Sterilization Procedures

Various sterilization procedures can be employed in the bacteriological laboratory. The method selected in each case depends on the stability of the culture media, the reagents, or the materials to be sterilized. The most common sterilization processes are: moist heat, dry heat, complete incineration, gas sterilization, filtration, UV radiation and irradiation.

The following sections discuss the more important aspects concerning sterilization of different laboratory materials.

7.2.1 Media and reagents

Tube culture media and reagents should be autoclaved at 121 °C for 10 to 12 minutes and not exceeding 15 minutes, unless other temperature or time are specified. The sterilization period should be counted from the time when the autoclave reaches 121 °C. Excessive exposure to heat may result in hydrolysis of sugars, specially lactose, and therefore will favor false positive reactions caused by noncoliform organisms.

Culture media for MF methods do not generally require autoclaving because exposure to sterilizing temperatures results in destruction of sodium sulfite in M-Endo media, and instability of some suppressive agents. However, exposure to heat is necessary to ensure complete dissolution of media ingredients. The most adequate heating method consists in placing the flasks of medium in a boiling water bath for 5

minutes. Since medium temperature reaches an approximately maximum of 97 °C during that period, the culture medium becomes sanitized.

7.2.2 Membrane filters and absorbent pads

Membrane filters are commonly sold presterilized and protected in individual envelopes; absorbent pads are usually packed in groups of 10 or more for easy dispensing into Petri dishes. When they are not presterilized, packets of membrane filters and absorbent pads must be sterilized by autoclaving at 121 °C for 10 minutes. Excessive exposure to sterilization temperatures may cause MF pores to seal, creating uneven water flow through the membrane, or cause membranes to become brittle and distorted. Since physical characteristics of nitrocellulose membranes change during storage, it is recommended that MF supplies should not be stocked beyond the estimated need for a 12-month period.

7.2.3 MF filtration equipment

Membrane filtration equipment should be cleaned and wrapped in kraft paper and then sterilized by autoclaving at 121 °C for 15 minutes. The wrapping should maintain sterility during laboratory storage. To sterilize at the laboratory bench between filtrations, the clean filter funnel and the membrane holder should be exposed to UV light for 2 minutes or submerged in boiling water for the same length of time.

The effectiveness of the UV disinfection can be measured by comparing colony counts on plates from the UV-exposed and unexposed suspensions; UV exposure should produce a 99% kill of the bacterial suspension employed.

Dry heat sterilization, by heating at 170 °C for 1 hour, can be used with glass filter assemblies, provided the rubber stopper is previously removed. However, metal or plastic units should not be sterilized using dry heat, due to rapid deterioration of neoprene or nylon lock wheels, or distortion of plastic material.

7.2.4 Sample bottle

The choice of sterilization method for sample bottles depends on whether plastic or glass bottles are used. Plastic bottles, and glass bottles with plastic caps, may be autoclaved repeatedly at 121 °C for 15 minutes. Screw caps should be let loosely closed to allow pressure equalization during autoclave sterilization. Glass bottles with ground-glass covers should be sterilized by dry heat, at 170 °C during 2 hours. This methods ensures complete drying of any dechlorinating agent solution that may have been added to the bottle before sterilization.

7.2.5 Glassware items

It is recommended that sterilization of individual glassware items commonly used in the bacteriological laboratory be conducted by dry heat method, by exposure to 170 °C for 1 hour. Metal foil or wrapped paper covers must be secured around open ends of these items to ensure they remain sterile during storage.

Glassware items such as pipettes and glass Petri dishes are generally stored in stainless-steel containers suitable for dry heat sterilization. To ensure adequate heat

penetration in these containers of glassware items, 2-hour exposure to dry heat at 170 °C is required. However, disposable hardwood applicators should only be dry heat sterilized because autoclaving may generate wood distillate products that may be toxic to bacteria.

7.2.6 Dilution water blanks

Dilution water blanks are sterilized by autoclaving at 121 °C for 15 minutes. Screw caps or rubber stoppers closures should be slightly loosened to allow pressure equalization during autoclaving. Some dilution water may be lost from either evaporation or boil-over when steam pressure is rapidly reduced during the autoclave exhaust cycle. Careful timing of the steam exhaust process should prevent this from happening. However, when volume losses of the dilution blank are consistently greater than 2 percent, 101-ml or 102-ml volumes of dilution water should be initially dispensed to compensate for the water losses during autoclaving. This adjustment will prevent pipetting sterile dilution water into deficient water blanks.

7.2.7 Culture dish reuse

Although plastic culture dishes used in the MF method are generally considered disposable items, they may be reused. The reuse method consists of discarding the old cultures and hand cleaning the top and bottom sections of the dishes in a mild household dish detergent. After rinsing and air-drying, the dish sections are ready for sterilization. Plastic material cannot withstand heat exposure during autoclaving, therefore other sterilization methods must be used. Plastic culture dishes can be sterilized by soaking individual top and bottom sections in 70% ethanol for 30 minutes, then placing them on a clean towel to drain and air dry before reassembly. A more convenient sterilization method is to expose the interior portions of the culture dishes to UV light for 5 minutes and reassembling them for storage or immediate use.

Plastic Petri dishes may also be sterilized with ethylene oxide, although control must be established to ensure no traces of the gas remain in the culture dishes.

To verify the efficiency of these sterilization methods, it is recommended to select one plate from each batch sterilized for use as a sterilization control. Standard plate count agar is added to the dish, mixed by gentle rotation, solidified, then incubated at 35 °C for 48 hours. No bacterial growth should appear on the control plate if sterilization was satisfactorily achieved.

7.2.8 Flame sterilization

Wire inoculating loops and needles are sterilized by heating them in an ordinary gas burner flame until the wire blows red hot. Needles and inoculating loops should be allowed to cool to near room temperature before using them, as to avoid killing the bacterial cells during transfer.

Forceps and spatulas are generally surface sterilized by dipping them in alcohol and then burning off the residual alcohol to incinerate any attached bacteria. Direct heating of forceps or spatulas may destroy the temper of the metal and may damaged the MF during manipulation.

7.3 Laboratory Water Quality

Water used in the bacteriological laboratory should be free of toxic or nutritive substances that could influence survival or growth of bacteria or viruses. Furthermore, the laboratory water supply should be free of microorganisms that may contribute inhibitory substances, or other undesirable substances, to dilution water.

The best distilled water system is one made of stainless-steel. However, adequate systems may also be built from glass. Polyvinyl chloride is a major contaminant in high-quality laboratory water systems and therefore should not be used for connecting piping.

A water treatment system consisting of a series of deionizing columns and a carbon filter can improve substantially the efficiency of the distillation process. However, these columns should be carefully maintained to prevent the released or breakthrough of organic and inorganic components previously removed from the tap water source.

Commercial systems are available that use disposable or rechargeable cartridges for prefiltering the source water, followed by organic adsorption, deionization, and finally membrane filtration. A reverse osmosis system, in conjunction with a series of deionizing columns, can also produce a water of excellent quality.

Electrical conductivity is an essential laboratory quality measurement, particularly when it is made at various points in the distilled water system. Although conductivity measurements reflect the presence of ionized material, they do not differentiate between the presence of toxic or nontoxic metal ions, or of organic contaminants that may be present. Specific chemical test will provide additional information for routine quality monitoring of the water. However, these analyses do not provide any indication of the relative biological toxicity of the impurities.

The biological toxicity or nutritive releases from distilled and deionized water supplies can be measured by a suitability test, as explained in Standard Methods (APHA, 1985). The suitability test should be performed annually, and after any equipment repair or cleaning of the water distribution network in the laboratory.

7.4 Dilution Water

Bacteriological examination of natural and polluted waters requires the use of serial dilutions of the water samples to obtain a bacterial density within the counting limits recommended for each analytical method. Ideally, the diluent should not alter the bacterial density of the water sample and should not reduce the recovery of attenuated organisms. The physiological state of the microorganisms to be recovered from a water sample greatly determines the proper choice of diluent. Therefore, the final choice of a suitable diluent for water samples must be based on the actual conditions of bacteria in the natural water samples to be analyzed, and not on the response of pure cultures of bacteria.

Distilled water is not recommended for water sample dilution because it is deficient in essential trace metal ions and in buffering and chelating capacities (Geldreich, 1975). Dilution of natural water samples with peptone water has showed that bacterial multiplications could occur at room temperature when the time between

sample dilution and inoculation exceeds 40 minutes. Therefore, when 0.1 percent peptone water is used as a diluent, a 30-minute limit on processing serial dilution must be closely followed. The pH of peptone dilution water should be adjusted to pH = 6.8.

To ensure that bacterial growth with minimal lag occurs when analyzing high quality natural waters, it is necessary to use a degree of mineralization corresponding to that of natural water. Phosphate-buffered dilution water satisfies adequately this requirement. However, sample processing through serial dilution must be completed within 30 minutes to prevent significant changes in the bacterial density at room temperature.

Stock potassium phosphate buffer solution should be adjusted to a pH = 7.2. The addition of magnesium sulfate to phosphate buffer dilution water improves the recovery of organisms with metabolic injury induced by high-quality water or by waters containing significant concentrations of heavy metal ions (MacLeod, 1967). The stock buffer solution should be discarded when turbidity due to microbial contamination is observed. Small portions of stock buffer solution should be prepared and, after sterilization by autoclaving at 121 °C for 15 minutes, be stored at 5 to 10 °C. In this manner, sterile stock buffer solution will be available in small volumes and, if chance contamination occurs, only a small volume of stock will have to be discarded.

8. CULTURE MEDIA SPECIFICATIONS

Most bacteriological laboratories use commercially prepared dehydrated media for routine water examinations, because of their reliability, convenience and labor-saving preparation. When the quantities of culture media required are small or the facilities and personnel of laboratories are limited, the use of prepared media available from commercial firms may be justified, although the unit cost is higher. Ampuled media or preweighed vials of dehydrated media may be used in a laboratory performing only a few tests and also when conducting analyses with portable MF kits, because of compact storage of media and the easier preparation under field operating conditions.

8.1 Media Preparation

Dehydrated culture media is commonly available as finely ground powders, granules or tablets. The choice among these preparations is mainly determined by cost, availability, quality control by the manufacturer and convenience. Finely ground powder is the form most frequently used. Regardless of their commercial form, the best method for preparing culture media consists in slowly adding the appropriate weighed quantities to approximately half of the total volume of distilled water. Media dissolution is aided by preheating the distilled water to approximately 45 to 50 °C. After thorough mixing, the remaining volume of distilled water is slowly added to wash residual powder from the inner walls of the container. Finally the culture medium is sterilized.

When the culture medium contains agar, the easiest method to dissolve the agar is to place the flask containing the ingredients in a boiling water bath until the agar medium dissolves into a uniform solution. An agar medium must be in complete solution before it can be dispensed into culture tubes or bottles.

Once a culture medium is dissolved, it should be dispensed into appropriate culture tubes or bottles and promptly sterilized by appropriate methods. To avoid bacterial growth in a culture medium, the time elapsed from media preparation to sterilization should not exceed 2 hours. The temperature of an agar medium should be maintained from 45 to 50 °C so that it remains fluid long enough to be adequately dispensed.

8.2 Media pH Measurements

The electronic pH meter used for preparing culture media must be calibrated in the pH range of intended use by means of a precision buffer standard. Since most bacteriological media used in the water laboratory are near pH = 7.0, the standard buffer chosen to calibrate the pH meter at daily intervals should be pH = 7.0. Colorimetric methods or pH paper strips are not acceptable for pH measurement because colour changes are masked by the dyes present in the culture media.

When the pH of the culture media deviates more than ± 0.5 pH units from the established tolerance limits, the pH meter should be checked immediately. If the pH meter is working properly, the preparation and sterilization methods should be carefully verified for possible errors. If the problem can not be traced to any of those factors, then the quality of the distilled water or that of the commercial culture medium should be suspected.

8.3 Media Storage

Dehydrated culture media do not remain stable indefinitely. These products undergo changes that can alter both their efficiency in culturing bacterial strains and their detection ability by biochemical reactions. Culture media supplies should be purchased in quantities estimated to last no longer than 1 year, and preferably no more than 6 months. No media should be purchased without express indication of its expiration date. Once a bottle of media is opened, it should be used within 6 months.

Once the culture media have been prepared, they should be stored in an area protected from direct sunlight, contamination, and excessive evaporation. Storage of sterile culture media over long time periods will increase the risk of contamination, fading of indicator colour intensity, precipitation or excessive evaporation; all of these factors can drastically affect the performance of the culture media prepared. Therefore, unless screw cap culture tubes or tight fitting culture dishes are used, culture media production should be limited to quantities calculated to be used within a 1-week period.

8.4 Media Quality Control

In general, using commercially prepared dehydrated media for routine analyses is preferable to preparing media from basic ingredients; commercial products are less subject to the variations in chemical composition that may be introduced when weighing individual components. Simple weighing of a preformulated medium should result in greater uniformity in composition and also reduce preparation time.

Although commercially prepared culture media are subjected to a quality control process, it may be inadequate at times. Commercial media containing sodium azide, such as M-Enterococcus and KF-Streptococcus, have an approximately shelf live of 2

years after production, because of the deleterious effects produced by the slow decomposition of the azide compound.

The analysis of a medium for selectivity and adequate quantitative recovery must be based on appropriate water samples, which can be altered by dilution or by dosing with selected organisms. The method for evaluating culture media is described in detail by Geldreich (1975).

8.5 Media pH Records

The pH of culture media should be measured after sterilization and recorded with the date and the medium lot number. As an absolute minimal requirement, the pH of at least one batch of sterilized medium from each new bottle of commercial medium must be measured to verify its quality. Measuring the final pH of culture media allows detection of errors that may have occurred during the preparation or sterilization of the media, or the possible deterioration experienced during storage of the dehydrated media since the packages were open.

8.6 Standard Culture Media Specifications

The following recommendations apply to culture media commonly used in the determination of total coliforms, faecal coliforms and faecal streptococci. The material safety data check and the hazard communication sheets included in each culture media container should be carefully read and their recommendations closely followed.

The Endo agar should not be sterilized at 121 °C for 15 minutes. Excessive exposure to heat destroys the sodium sulfite present in the culture medium, resulting in poor sheen development in coliform colonies. Therefore, the agar preparation should be dissolved in a boiling water bath, cooled to 45 °C, and then poured in an adequate number of culture dishes.

Excessive exposure to heat of M-Endo medium destroys or reduces its specificity. Therefore, the medium should only be heated to the boiling point, as described in the sterilization sections. The amount of M-Endo medium prepared should be adequate to meet anticipated daily needs. However, surplus medium may be used within a 96-hour period provided it is stored in the dark at 2 °C. Storage in the dark is essential since M-Endo medium is sensitive to strong artificial light or direct sunlight.

Aniline blue is included in the M-FC medium as the indicator for detecting lactose fermentation. Development of the blue colony colour does not depend upon the addition of the rosolic acid salt reagent. By adding the sodium salt of rosolic acid to the M-FC medium a variety of nonfaecal coliform organisms are suppressed. Without the inhibitory effect of the rosolic acid salt, a substantial background growth of white and gray-coloured colonies may develop and interfere with the distinct blue colonies of faecal colonies. The addition of rosolic acid is facultative and may be omitted from the M-FC broth if minimum background colony counts occur and equivalent results are obtained without it.

The amount of M-FC broth prepared should be adequate to meet anticipated daily needs. However, surplus medium may be used within a 96-hour period provided it is stored in the dark at 2 to 10 °C.

M-Enterococcus agar and KF-Streptococcus agar should be heated in a boiling water bath to dissolve the agar. After solution is complete, continue heating for an additional 5 minutes. When preparing KF-Streptococcus, the medium should then be cooled to between 45 and 50 °C, and 1 ml of sterile aqueous 1 percent solution of tripheniltetrazolium should be added per each 100 ml of medium. M-Enterococcus agar already includes this compound. The pH of the final medium should be adjusted to 7.2 with 10 percent sodium carbonate, if necessary. The fluid agar may be stored up to 4 hours in a water bath at 45 to 50 °C, until it is dispensed. The M-Enterococcus agar and the KF-Streptococcus agar employed with the MF method may be used immediately or stored in a cool and dark place, and used within 4 weeks, provided no dehydration has occurred.

9. THE MULTIPLE TEST TUBE METHOD

The multiple test tube method for enumeration of total coliforms, faecal coliforms and faecal streptococci are explained in detail in the relative Reference Methods for Marine Pollution Studies prepared by WHO within the framework of the MED POL Programme. In this method, replicate tubes of the appropriate culture medium are inoculated with decimal dilutions of a water sample. The bacterial densities are then calculated from probability formulae that predict the most probable number of bacteria necessary to produce the combinations of positive and negative tubes actually observed when analyzing replicate decimal dilutions. The Most Probable Number (MPN) is obtained from the corresponding MPN tables, as presented in the Reference Methods and Standard Methods (APHA, 1985).

9.1 Total Coliform Method

The determination of total coliforms includes three distinct test stages: the presumptive test, the confirmed test, and the completed test. The objective of the presumptive test is to stimulate the metabolic activity of attenuated bacteria and to achieve a gross selection for lactose-utilizing organisms. After incubation at 35 °C, a small inoculum of cultures from each gas-positive presumptive tube is transferred into a tube of medium for the confirmed test. The objective of the confirmed test is to reduce the possibility of false gas-positive results. To verify that the confirmed test selectively eliminates all false positive results, it will occasionally be necessary to isolate these gas-producing bacteria and identify them as coliforms by the completed test procedure.

The choice of dilutions to be used in the multiple tube test must be based on the information supplied with the water sample. Results previously obtained at the same or similar water sampling stations will also help in deciding what dilution level to use in inoculating culture tubes.

Water samples must be shaken vigorously immediately before removing aliquot sample to inoculate a series of presumptive tubes in the multiple tube test. Inadequate mixing will definitely result in heterogenous distribution of bacteria, specially when analyzing turbid water samples. As a result, the combinations of gas-positive results will be inconsistent and the Most Probable Number of bacteria will be quite erroneous.

Presumptive test tubes should be read after 24 ± 2 hours. Each tube should be examined carefully. Tubes showing gas in the fermentation vial are recorded as positive,

promptly submitted to the confirmed test, and then discarded. Gas in any quantity is recorded as positive. All positive tubes must be confirmed at the end of the initial 24-hour period regardless of the amount of gas produced. Since gas positive tubes contain a mixed bacterial population competing with coliforms, it is possible that tubes containing coliforms, and showing gas production after 24 hours, may give negative results when confirmed after incubation for 48 hours.

The two broths used in the presumptive test are lactose broth and lauryl tryptose broth, and they yield equivalent recoveries of coliform organisms. However, lauryl tryptose broth inhibits the development of aerobic spore-forming organisms that often ferment lactose with gas production. Comparative analyses using lactose broth and lauryl tryptose broth may reveal a significant reduction in false positive presumptive tubes with the use of lauryl tryptose broth. Therefore, the choice of presumptive medium should be based on a comparative evaluation of a variety of water samples normally examined in the laboratory.

Culture tubes with no gas production are recorded as negative and returned for an additional 24-hour incubation period. These cultures are then inspected for evidence of gas production or heavy growth. Positive tubes are submitted to the confirmed test, and those with no gas production or little growth are recorded as negative and discarded.

Although gas production in the presumptive test indicates the probable presence of coliform bacteria, gas may be produced by other organisms. Therefore, to confirm the presence of coliform bacteria all positive presumptive tubes should be submitted to a more selective test after enrichment in lactose or lauryl tryptose broth. The confirmed test consists of transferring a small inoculum of culture from each positive presumptive tube, and also from those with heavy growth, to individual broth tubes containing brilliant green lactose broth (BGLB) and incubating them at 35 °C for 48 hours. Gas production in the confirmed tubes indicates that coliform bacteria are actually present in the water sample examined.

The completed test is the reference standard for the multiple tube method. Since the confirmed test may yield positive reactions in the absence of the coliform group (false-positive test), it is recommended that periodic comparisons be made between the completed test and the confirmed test to verify data reliability. A series of 5 test per month should be sufficient when good agreement between the two tests is observed. The completed test is applied to all gas-positive confirmed tubes in the individual test. The detailed method for the complete test for total coliforms appears in the corresponding recommended Method in Part II of these guidelines.

9.2 Faecal Coliform Method

The determination of faecal coliforms can be performed by an additional step of the multiple tube method for total coliforms. A small inoculum of culture from each positive tube of the presumptive test is transferred to a corresponding tube of EC broth. EC broth tubes are incubated at 44.5 ± 0.2 °C for 24 hours in a covered water bath to ensure optimum temperature regulation.

EC broth tubes showing gas production after the 24-hour incubation period are considered as positive. Any direct inoculation of aliquot samples into EC tubes without preliminary enrichment in either lactose or lauryl tryptose broth is unsatisfactory. Furthermore, incubation of 1x broth culture tubes beyond the 24-hour period is not recommended since there is a very minor probability that changes in gas reaction may occur.

9.3 Faecal Streptococci Method

The multiple tube method for enumeration of faecal streptococci employs azide dextrose broth for the presumptive test. Inoculated tubes are incubated at 35 ± 0.5 °C for 24 hours, and subsequently examined for the presence of turbidity. If no definite turbidity appears after 24 hour of incubation, the tubes are reincubated and read again at the end of 48 ± 3 hours.

All culture tubes showing turbidity after the 24 or 48 hours incubation period are subjected to the confirmed test. A small inoculum of culture from each positive azide dextrose broth tube is streak on a Petri dish containing esculin-azide agar. The culture dishes are then incubated at 35 ± 0.5 °C for 24 hours. The presence of brownish-black colonies with brown halos will confirm the presence of faecal streptococci. Although the test is designed primarily for raw wastewater and chlorinated wastewater effluent samples, it is applicable to other fresh, but not saline waters. This method is commonly used on waters with high turbidities, because of the interferences with the membrane filter.

9.4 Calculations of the Most Probable Number

The most probable number (MPN) of bacteria in a water sample can be statistically estimated from the number of positive and negative results obtained in the multiple tube test. The multiple tube combinations most frequently used are the five replicate portions in three decimal dilutions, although the three replicate samples can also be used. The greater the number of replicates of each sample volume in a dilution series, the greater the test precision.

When monitoring marine water samples, a five-tube, three decimal dilutions MPN should be used to obtain a more accurate coliform enumeration (UNEP/WHO, 1985a, b, c). The practice of using a three-tube, rather than a five-tube combination, results in a MPN density significantly less precise, as illustrated by the larger 95% confidence interval corresponding to the three-tube procedure. Furthermore, the MPN tables were originally calculated to include a positive bias for health safety reasons. Taking this bias into consideration, the values reported for a three-tube MPN test may be overestimated by a factor of 43 %, whereas the corresponding values using the five-tube MPN test are overestimated by only 23% (Geldreich, 1975).

The MPN tables commonly used contain the positive tubes combinations most likely to occur during three subsequent dilutions inoculated in series of three or five tubes each. However, for special studies involving other combinations of replicate tubes and dilutions, a simple approximation of the MPN value may be obtained using the short formulas indicated in Standard Methods (APHA, 1985). Each positive tube combination has an estimated frequency of occurrence when performing routine bacteriological analyses. When the frequency of the MPN positive tube combinations

recorded exceed those associated to the less frequent combinations, the multiple tube method is probably in error. Such abnormal results may be due to the presence of inhibitory substances in the water samples, or improper laboratory procedures.

10. MEMBRANE FILTRATION METHOD

10.1 Introduction

The membrane filtration (MF) method for enumeration of total coliforms, faecal coliforms, and faecal streptococci are explained in detail in the relative Reference Methods for marine pollution studies. These methods involve filtering a known volume of water sample through a MF of optimum pore size for full bacterial retention. As water passes through the pores, bacteria are entrapped on the surface of the MF. The MF is then placed in contact with either a paper pad saturated with liquid medium or directly over an agar medium to provide nutrients for bacterial growth. After incubation under prescribed conditions of time, temperature, and humidity, the MF cultures are examined for bacterial colonies of specific characteristics that are then counted and recorded as a bacterial concentration per 100 ml of water sample.

The presence of high concentrations of suspended matter in turbid waters, and that of high concentrations of noncoliform bacteria are two of the main limitations of the membrane filter methods. In those cases, the MPN is the recommended method for bacteriological analysis of water samples.

The MF method may be adjusted to promote recovery of attenuated coliform bacteria. The method frequently used consists in a two layer enrichment differential growth medium that allows for repair and subsequent reproduction of those faecal coliforms that have been stressed by exposure to chlorine, industrial wastes or marine waters. General procedures and considerations regarding recovery of stressed indicator organisms appear in Standard Methods (APHA, 1985).

The decision to use the double layer M-FC agar method or any other coliform MF method for the bacteriological assessment of coastal water quality must be based on comparative analyses that show at least an 80 percent agreement between parallel MF and MPN faecal coliform methods. Approximately 100 samples chosen from a variety of coastal sampling stations should be used in this MF-MPN comparative study.

10.2 Total Coliform Method

Proper application of MF methods requires development of good laboratory and routine operational practice. Since the MF method is quantitative, water samples should be measured within a ± 2.5 percent tolerance as specified in the MF methods. Graduated cylinders or volumetric pipettes should be used for accurate measurement of water sample volumes. An individual, sterile, graduated cylinder or volumetric pipette should be assigned to each sample examined in the filtration series.

Sample bottles should be shaken vigorously immediately before obtaining the water sample to be filtered. This vigorous shaking is needed to ensure an homogeneous distribution of bacteria and is particularly critical when filtering very turbid water. After shaking the sample thoroughly, pour or pipette the measured sample

volume into the funnel with the vacuum supply line connection turned off. To avoid uneven distribution of organisms over the effective filtering area, the vacuum should never be applied simultaneously with the addition of the sample test portion. When dispensing 10 ml or less, approximately 20 ml of sterile dilution water should be previously added to the funnel to ensure uniform dispersion of the bacterial suspension. The vacuum should then be applied to force the passage of the water through the MF, and then the funnel should be rinsed with 20-30 ml of sterile dilution water. After the first rinse has passed through the filter, the rinsing procedure should be repeated. With a proper rinsing technique, bacterial retention on the funnel walls is negligible.

To ensure that no contamination exits at the beginning of the analysis, a sterile 100-ml dilution blank should be analyzed by the MF method, before processing any other water sample. Water samples should be analyzed in increasing degree of bacterial content. One sterile water sample should be analyzed at the conclusion of each different groups of waters and one at the conclusion of the filtration series. The purpose of this quality control procedure is to ensure that funnel assemblies are sterile at the start of filtration, and to detect possible cross-contamination if the technician fails to adequately rinse all organisms onto the filter surface of a polluted sample.

10.3 Incubation and Counting of MF Cultures

MF examination for total coliforms recovery requires a 22 to 24-hour incubation period at 35 °C for optimum growth and sheen development. A typical coliform colony has a pink to dark red colour with a metallic surface sheen. All members of the coliform group grow and develop a metallic sheen on Endo-type media. Noncoliform colonies vary in appearance from colourless to a deep red colour. Colonies having a red colour and a touch of shiny material resembling a metallic sheen are the most confusing of the noncoliform types and are responsible for the overestimated MF coliform counts made by inexperienced bacteriologists. The bile salts present in Endo-type media are inhibitory to injured coliforms and thus a resuscitation step may be necessary to recover stressed coliforms that may be present in coastal marine waters.

The coliform concentration may be calculated from one or more MF counts obtained from analyzing serial sample portions, provided the counts are in the 20 to 80 colony range and the total count of all colonies in the MF does not exceed 200. When different volumes of sample are examined, it is permissible to total the counts on each membrane and base the value on the total volume of sample examined. When monitoring coastal waters, an attempt should always be made to quantify the number of colonies grown in the MF. If all the cultures prepared from a water sample exceed the maximum recommended limit, the bacterial count could be estimated from a partial enumeration of the MF with the lower colony count.

When confluent growth occurs, that is, growth covering either the entire filtration area of the membrane or a portion thereof, and colonies are not discrete, report results as "confluent growth with (or without) coliforms". If the total number of bacterial colonies, coliforms plus noncoliforms, exceed 200 per membrane, or if the colonies are not distinct enough for accurate counting, report results as "too numerous to count" (TNTC) (Standard Methods, APHA, 1985).

The M-Endo broth can be transformed to an agar form by adding 1.5 percent agar. Results from the intercalibration exercises conducted in the Mediterranean (UNEP,

1986) revealed the interest of using M-Endo agar, thus eliminating the need for an absorbent pad.

Inexperienced technicians frequently find the deep red colonies difficult to classify, especially when the presence or absence of a metallic sheen is the only distinguishing characteristic. The proper identification of coliform colonies by a new technician can only be solved by actual practice and experience in counting colonies, together with the use of the confirmed test. This verification procedure should be followed by any laboratory technician suspecting that typical sheen colonies may not be coliform bacteria.

Coliform colonies can be confirmed by transferring them to lactose or lauryl tryptose broth and then to Brilliant Bile Lactose broth BGLB for evidence of gas production at 35 °C within the 48-hour limit.

10.4 Faecal Coliform Method

The MF method for faecal coliform enumeration requires: 1) a water bath which can be regulated at 44.5 ± 0.2 °C, 2) M-FC culture media, and 3) sealable plastic or metal containers to protect the cultures while immersed in the water bath incubator. For specific details on the MF method, the corresponding Reference Method for marine pollution studies should be consulted.

The faecal coliform concentration may be calculated from one or more MF counts obtained from analyzing serial sample portions, provided the counts are in the 20 to 60 colony range. When different volumes of sample are examined, it is permissible to total the counts on each membrane and base the value on the total volume of sample examined. When monitoring coastal waters, an attempt should always be made to quantify the number of colonies grown in the MF. If all the cultures prepared from a water sample exceed the maximum recommended limit, the bacterial count could be estimated from a partial enumeration of the MF with the lower colony count and the remark of "confluent growth" should be indicated.

The M-FC broth can be transformed to an agar form by adding 1.5 percent agar. Results from the intercalibration exercises conducted in the Mediterranean (UNEP, 1986) revealed the interest of using M-FC agar, thus eliminating the need for an absorbent pad.

Gray to cream-coloured colonies may be occasionally observed on M-FC cultures. These organisms are not faecal coliforms and should not be counted as such. M-FC cultures should be counted promptly after their removal from the incubator, since exposure to room temperature for more than 30 minutes may allow some of the nonfaecal coliform colonies to ferment enough lactose to develop a pale blue colour.

10.5 Faecal Streptococci Method

The MF method for enumeration of faecal streptococci requires a 48 hours incubation at 35 °C for optimum growth. Colonies produced by faecal streptococci are dark red to pink. Counting should be done using a 10x to 15x magnification microscope or similar optical instrument.

The MF method for faecal streptococci has ben recommended over the corresponding multiple tube method for the following reasons: 1) recoveries on MF media are higher and less influenced by interference organisms, 2) the MPN methods result in greater numbers of false positive reactions, and 3) when identification is required, MF plates allow for primary isolation of faecal streptococci colonies. However, the comparative evaluation on MPN and MF methods carried out during the intercalibration exercises conducted in the Mediterranean offered no significant difference between the two methods, particularly when using the M-Enterococcus agar (Mujeriego, 1986).

The two media commonly used for the MF method are the M-Enterococcus agar and the KF-Streptococcus agar. Although both media are considered to give similar results in wastewater samples, studies conducted in various Mediterranean laboratories have shown a considerable number of false positive colonies when using the KF-Streptococcus agar for the analysis of coastal water samples (Yoshpe-Purer, 1988; Feliu, 1988). Therefore, the M-Enterococcus agar has been proposed as a more adequate media, although it may underestimate the faecal streptococci content of water samples (UNEP, 1979a). To verify the colonies grown on the membranes filters, a confirmed test should be conducted.

The faecal streptococci concentration may be calculated from one or more MF counts obtained from analyzing serial sample portions, provided the counts are in the 20 to 100 colony range. When different volumes of sample are examined, it is permissible to total the counts on each membrane and base the value on the total volume of sample examined. When monitoring coastal waters, an attempt should always be made to quantify the number of colonies grown in the MF. If all the cultures prepared from a water sample exceed the maximum recommended limit, the bacterial count could be estimated from a partial enumeration of the MF with the lower colony count and the remark of "confluent growth" should be indicated.

Normally, there is no need for species identification of faecal streptococci in coastal water quality studies. The relationship between faecal coliforms concentration and faecal streptococci concentration in a fresh wastewater sample is a useful parameter for establishing the probable source of the waste discharge as being domestic or from farm animals or wildlife. However, the more resistant character of faecal streptococci in sea water results in a decreasing value of that relationship regardless of the wastewater source. In these cases, the faecal coliforms to faecal streptococci ratio, or the faecal coliforms to faecal enterococci ratio, can be better interpreted as a measure of the distance of the sampling station to the waste source, or of the time elapsed for the arrival of the wastewater to the coastal sampling point.

10.6 Replicate Samples

The colony count obtained by the MF method is not an absolute exact value, but rather an estimation of the actual bacterial density of the water sample examined. By increasing the number of replicate analyses, the 95 percent confidence interval of the average MF count will be narrower around the true bacterial density of the water sample. Thus for routine analyses, it is recommended to perform a replicate sample approximately every tenth sample to verify continued level of data precision.

11. INTERPRETATION OF RESULTS

11.1 Introduction

Conformity of a water sampling station with the microbiological limits established by coastal water quality criteria and standards usually requires determination of the microbial concentrations not exceeded in a given percentage of the water samples analyzed. A subsequent comparison between those percentiles and the microbial concentrations set forth by the criteria or standards allows an evaluation of the microbiological quality of the sampling station considered.

Although most microbiological criteria and standards for coastal waters are expressed in terms of two concentrations of a specified bacterial indicator which should not be exceeded in two corresponding percentages of the samples analyzed, very few criteria or standards include explicit indications on how to obtain the microbial concentrations characteristics of a set of experimental results.

As an illustration, the interim criteria for microbiological quality of recreational waters approved by the Contracting Parties to the Barcelona Convention (UNEP, 1985) specifies that "the faecal coliforms concentrations of at least 10 water samples collected during the bathing season should not exceed: a) 100 faecal coliforms per 100 ml in 50% of the samples, and b) 1000 faecal coliforms per 100 ml in 90% of the samples" The interim criteria recommend a graphical or analytical adjustment to a lognormal probability distribution as the method for interpreting the results.

The two methods commonly used for evaluating the experimental results obtained at a given sampling station are 1) the ranking method and 2) the lognormal probability method. The following sections include a brief discussion of these two methods, whose detailed application can be found in the corresponding Reference Method for marine pollution studies (Mujeriego, 1982).

11.2 The Ranking Method

A method frequently used for assessing the conformity of a water sampling station with the corresponding quality criteria or standards consists in ranking the experimental values, in increasing order, and then selecting the microbial concentrations corresponding to the percentiles set forth in the standard or criteria. The rank number of those percentiles are obtained by multiplying the total number of experimental values by the percentages specified by the criteria or standards. The water sampling station will conform to the relevant quality criteria or standard when the bacterial percentiles obtained do not exceed the bacterial limits set forth in the criteria or standard.

As an illustration, the assessment of a sample station with 20 faecal coliforms concentrations would require ranking the experimental values in increasing order and then selecting the concentrations associated to the rank numbers n50 = 20 x 0.50 = 10 and n90 = 20 x 0.90 = 18. The water sampling station will conform to the WHO/UNEP interim quality criteria if the microbial concentrations associated with those rank numbers do not exceed the two bacterial limits indicated in the previous section.

The main characteristics of the ranking method are:

1. It is very simple to perform, as it does not require the use of complex formulas or graphical analyses.

2. It frequently leads to the practical difficulty of having to interpret rank numbers that are not integers, as for example when calculating n90 from a total of 14 values. This difficulty is usually solved by some rounding-off criterion, or by requiring a total number of samples that prevents this situation from occurring. This later condition brings considerable operating and interpretation problems.

3. The precision of the percentile microbial concentration is quite variable and relatively low.

4. The method takes only into account the absolute values of the percentiles specified by the criteria or standard, disregarding the remaining experimental results.

5. It does not provide any insight into the temporal variation of the microbiological quality of the water at the sampling station considered.

11.3 The Lognormal Distribution Method

The lognormal distribution method is based on the observed property of the microbial concentrations measured at a sampling station to follow a lognormal probability distribution. This method requires determination of the lognormal distribution that most closely fits the natural logarithms of the experimental results. The adjustment procedure can be performed either graphically or numerically, both alternatives being capable of producing identical results provided the calculation steps are adequately specified.

The main characteristics of the lognormal probability method are:

1. It requires some knowledge of geometry and certain skills in graphical treatment of data. However, the method can be programmed for simple application in personal and mainframe computers.

2. There are no practical difficulties due to the total number of experimental results available. Although the benefits of this evaluation method become more evident with increasing number of results, any set of experimental values can be evaluated.

3. The precision of the method can be statistically estimated and is generally higher than that of the ranking method.

4. The method takes into account the values of all the microbial concentrations considered.

5. The method estimates the lognormal probability distribution that most closely fits the experimental results and thus provides very helpful insight into the temporal variation of the water quality, and allows comparison between different sampling stations.

The simplest way of applying the lognormal probability method is by means of a lognormal probability paper. By adequately drawing the experimental results and visually interpolating the data points it is possible to estimate the lognormal probability distribution that best fits the bacterial concentrations. The systematic evaluation of the microbiological quality of coastal water during the two phases of the MED POL Programme, using the lognormal probability method, have shown the adequacy and interest of this method (Mujeriego, 1983). Only in very few sampling stations was the probability model not applicable, and even then the graphical representation of the data offered some clues as to the more adequate alternative method for evaluating their water quality.

Figure 1 illustrates the use of the lognormal probability paper for interpreting the microbiological quality of a coastal sampling station. Direct comparison of the probability distribution with the two limits set forth in the Mediterranean interim criteria allows the evaluation of the sampling station according to those quality criteria.

A systematic application of this interpretation and evaluation method to microbiological results obtained during the MED POL Programme should provide a more reliable and consistent assessment of the microbiological quality of coastal waters in the Mediterranean.

12. ANALYTICAL LABORATORY QUALITY CONTROL

12.1 Introduction

Among the elements of a quality assurance programme, the following three are directly related to the analytical quality control of laboratory activities: 1) the use of standard operating procedures, as indicated in the corresponding Reference Methods for marine pollution studies prepared by UNEP, or in Standard Methods (APHA, 1985), 2) the systematic analysis of control samples with unknown microbial concentrations, and 3) the periodic analysis of a few reference samples prepared by some central laboratory; as water samples with known microbial concentrations are difficult to prepare and preserve, replicate samples of unknown microbial concentrations are commonly used for distribution to participating laboratories.

The second quality assurance element mentioned above is designated intra-laboratory or statistical quality control, and the third is known as external laboratory quality control, inter-laboratory quality evaluation, or intercalibration of analytical laboratories.

The following sections describe some basic techniques commonly used for intra-laboratory quality control and inter-laboratory quality evaluation.

12.2 Internal Quality Control

Control charts are the techniques most frequently used for internal quality control of analytical laboratories. Their applicability is based on the assumption that experimental results approximately follow a normal distribution. Although this is usually an acceptable hypothesis in chemical laboratory practice, the microbial concentration of a water sample has to be numerically transformed to obtain a normal variable. By calculating the logarithm of the microbial concentration, a new value is obtained that more closely behaves as a normal variable, allowing the application of this process control technique to the statistical control of microbiological laboratories.

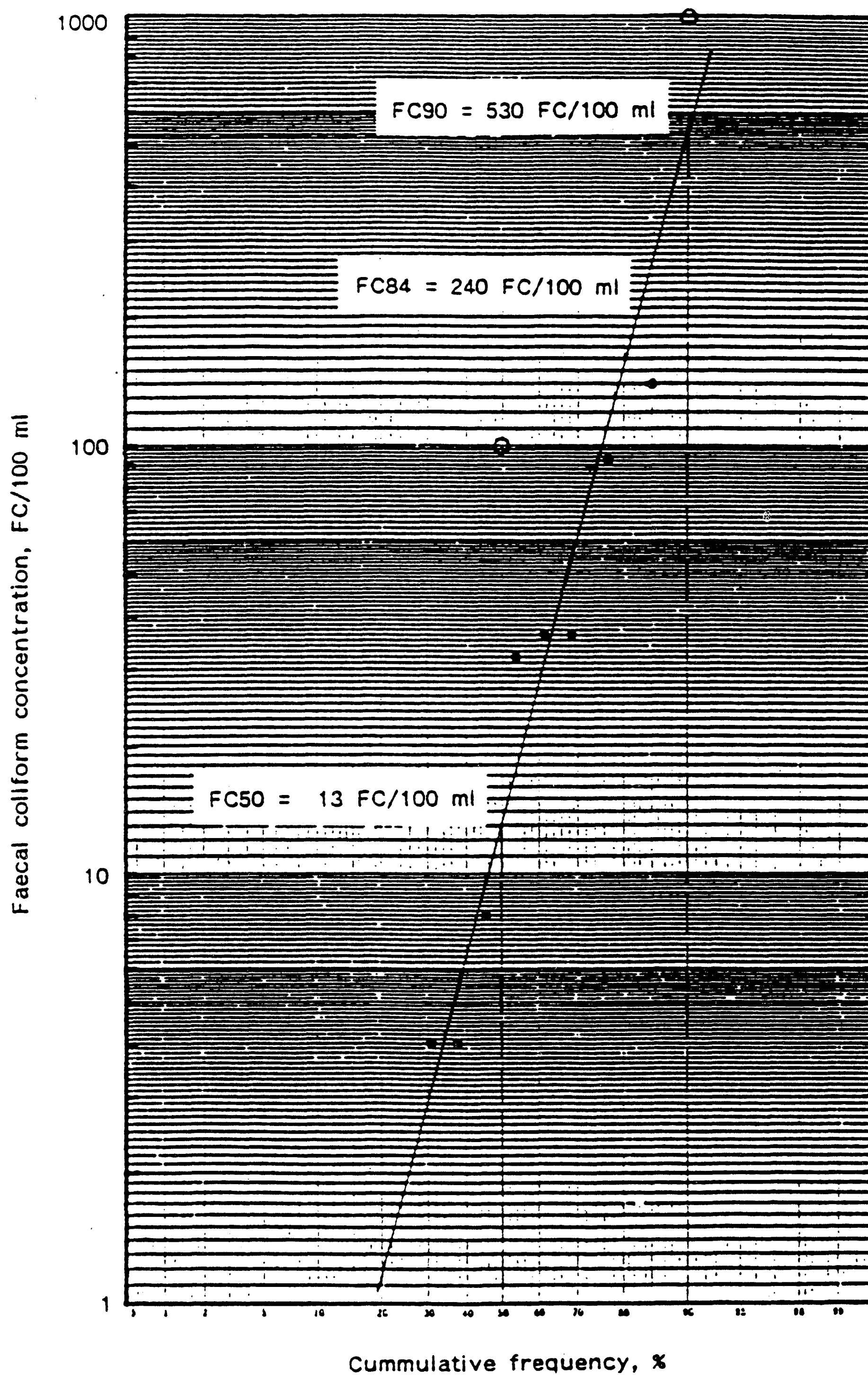

Figure 1. Interpretation of the microbiological quality of a coastal sampling station in the Mediterranean Sea by the lognormal probability model, and evaluation according to the interim environmental criteria adopted by the Contracting Parties (UNEP, 1985).

Several types of control charts can be prepared. The two most commonly used in statistical quality control of analytical laboratories are: 1) the X or average chart, to evaluate the precision of replicate analyses of a given water sample, and 2) the R or range chart, to evaluate the precision of replicate analyses of different water samples. In contrast to what happens with chemical solutions, the bacterial content of a water sample does not remain stable with time, rendering quite difficult replicate analyses over more than a few hours. As a result, the R charts are those normally used to evaluate the analytical precision of microbiologists examining water samples of variable bacterial concentrations.

The fundamentals and practical applications of control charts are described in specialized references such as Statistical Quality Control (Grant and Leavenworht, 1980) and Standard Methods (APHA, 1985). Table 1 and Figure 2 illustrate the application of the R control chart to the experimental results obtained during the intercalibration exercise conducted among microbiological laboratories in Catalonia (Mujeriego, 1985).

Table 1 indicates the concentration range calculated from 5 replicate analyses conducted on three bacterial indicators during a 8-week period. The values appearing in Table 1 were obtained as the difference between the higher and the lower concentrations of each data set, expressed in decimal logarithm. Although it is recommended that for best results a series of at least 20 pairs of duplicate determination should be obtained, the shorter series of results was compensated in this particular case by conducting 5 replicates determinations, a value much higher than those commonly used.

Figure 2 illustrates the evolution of the concentration range with the different water samples analyzed. The experimental data points fall within the upper control limit and the lower control limit of the charts for the three microorganisms, with the exception of the total coliforms range for sample number 4. These results indicate that the analytical precision of the microbiologist remained within acceptable limits, in all but one series of total coliforms replicate analyses. The systematic application of this statistical technique ensures that the analytical precision is kept within acceptable limits and provides an immediate indication of possible sources of errors within the analytical procedure.

It is important to realize that the R charts appearing in Figure 2 do not provide any indication of the accuracy of the analytical procedure, for this would necessarily require knowledge of the true microbial concentration of the samples, an information seldom available in practice.

The R control charts of Figure 2 indicate that, under stable conditions of the analytical procedure conducted by the specific microbiologist, it can be expected that the membrane filter method may result in R values as high as 0.53 in the analyses of faecal streptococci. This represents a ratio of 3.40 between the highest and the lower values of a set of 5 faecal streptococci determinations. Similarly, the ratio between the highest and the lowest of the faecal coliforms concentrations may be expected to be as high as 2.40. These values clearly illustrate the considerable different bacterial concentrations that one microbiologist may find when analyzing a water sample according to standardized procedures.

Table 1

Concentration range, expressed in terms of decimal logarithm, of the total coliforms, faecal coliforms and faecal streptococci obtained by a single microbiologist when conducting 5 replicate analyses of water samples by the membrane filter method (Mujeriego, 1985).

DATE	CONCENTRATION RANGE, R		
	TC	FC	FS
05.07.1983	0.30	0.12	0.14
12.07.1983	0.12	0.05	0.20
19.07.1983	0.24	0.08	0.07
26.07.1983	0.57	0.25	0.27
02.08.1983	0.15	0.30 (*)	0.40
09.08.1983	0.09	0.25	0.34
16.08.1983	0.13	0.24	0.36
23.08.1983	0.10	0.14	0.23
Average R	0.21	0.18	0.25
Upper control limit UCL = D_4 x R D_4 = 2.115	0.45	0.38	0.53
Lower control limit LCL = D_3 x R D_3 = 0	0.00	0.00	0.00
(*) Obtained from 3 replicate analyses, as the other 2 only contained one coliform colony each			

12.3 External Quality Control

Intercalibration of analytical laboratories has become an essential element of quality assurance programmes aimed to ensure that results obtained by different laboratories are reliable and comparable. External quality control has made considerable progress in chemical laboratories through the use of reference and standard samples that remain stable for extended periods of time. The MED POL Programme has played an important role in providing and analyzing reference samples of organic compounds and heavy metals. Microbiological laboratories have not been able to reach similar levels of external quality control due to the practical difficulty of preparing and maintaining stable water samples of known bacterial concentration.

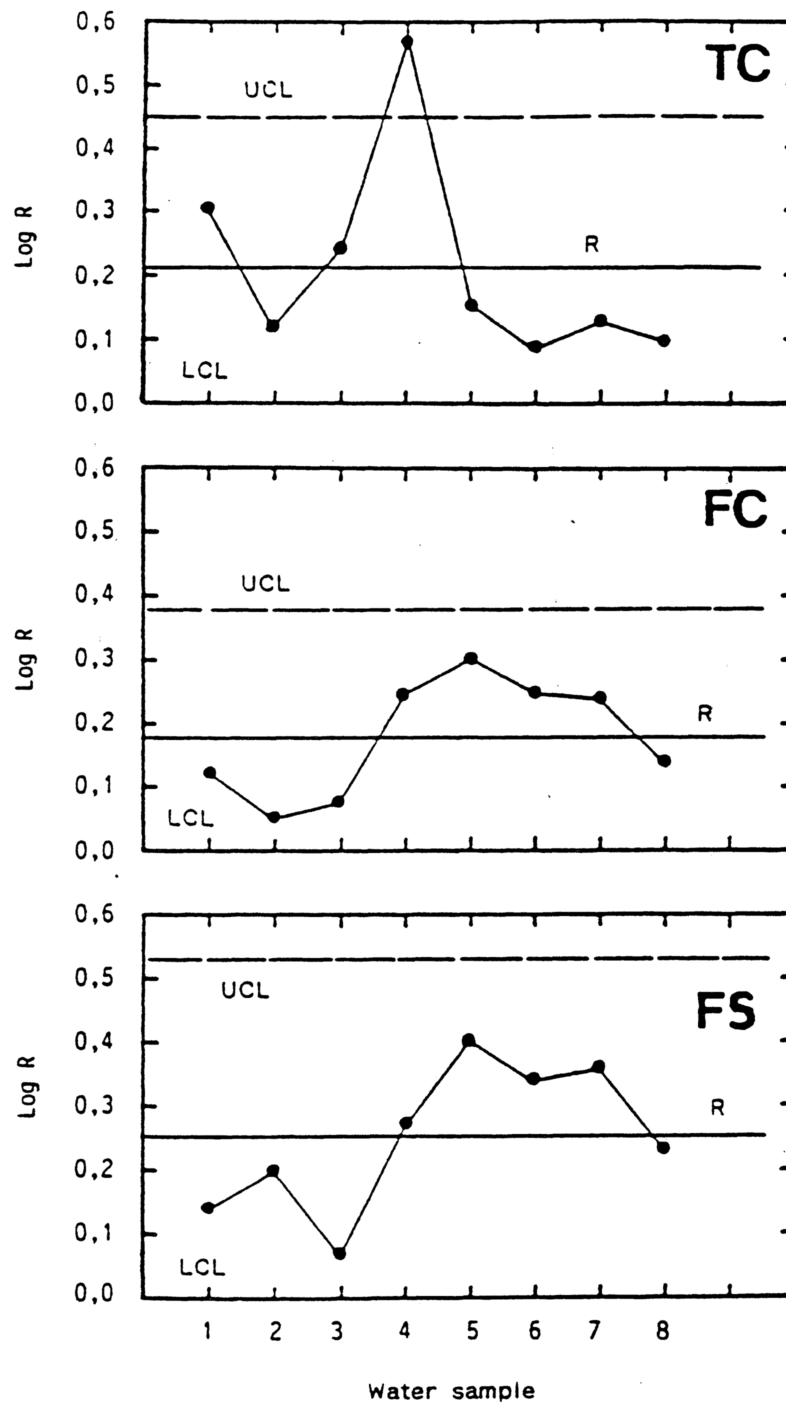

Figure 2. Control charts of the concentration range of total coliforms, faecal coliforms and faecal streptococci obtained by a single microbiologist when conducting 5 replicate analyses of water samples by the membrane filter method (Mujeriego, 1985).

Attempts have been made to overcome this limitation by preparing replicate natural water samples and distributing them to different microbiologists working in one given laboratory or, in some cases, sending the samples to different laboratories for immediate analysis. The fist approach was adopted during the 5 intercalibration exercises conducted in the Mediterranean region during the 1982-85 period.

Although this form of external quality control did not take into account the analytical conditions of each participating laboratory, it greatly contributed to harmonize analytical methodologies, to evaluate the comparability of the MPN and MF methods, and to point out the training needs among Mediterranean microbiologists (UNEP/WHO, 1986). As a results of these intercalibration exercises, the recommendation was made (UNEP/WHO, 1986, 1989) to carry out an external quality control among Mediterranean laboratories as the best way to ensure the comparability and reliability of their results.

Few attempts have been made to conduct an external quality control programme in which replicate water samples are sent simultaneously to different microbiological laboratories for their analysis. One of these was conducted in Catalonia, Spain (Mujeriego, 1985). The results of this intercalibration exercise, conducted among 11 microbiological laboratories of Catalonia, will be used to illustrate some of the alternative ways in which the experimental results can be interpreted and evaluated.

The interpretation of the basic microbiological results obtained in this intercalibration exercise was carried out by statistical methods based on the normal behaviour of the natural logarithm of the microbial concentrations. Two basic evaluation processes were applied: 1) an evaluation of the relative precision of the individual microbial concentrations obtained by each laboratory for each water sample, and 2) an evaluation of the water quality assessment obtained by each laboratory for the sampling station considered, over the whole study period.

Water samples were systematically collected at a coastal sampling station, distributed into replicate portions, packed adequately with chemical ice packages, and sent to participating laboratories for reception within 4 hours after sampling. The water samples were analyzed by the MF method for total coliforms, faecal coliforms, and faecal streptococci. Relative precision was always established by comparison to similar results obtained at a central reference laboratory.

12.3.1 Evaluation of microbial concentrations

Figure 3 illustrates the evolution of the faecal coliforms concentration obtained at the reference laboratory on series of replicate water samples analyzed at different times after collection. The experimental points show a relative decrease of the precision of the analytical method as the time elapsed after sampling goes beyond 23 hours. While analyses performed within 9 hours after sampling are generally centered around the regression line, the results obtained from analyses conducted after 23 hours of sampling show a larger scattering from that line.

Furthermore, the regression line in Figure 3 indicates that analyses conducted after 10 hours of sampling collection represent a 25% mean reduction on the faecal coliforms concentration. However, the considerable high value of the standard deviation associated to samples analyzed at a given time results in a 95% confidence interval equivalent to faecal coliforms concentrations from 0.25 to 2.00 times the initial

concentration. These results further illustrate the interest of a statistical evaluation of microbiological results.

Considering the precision achieved at the reference laboratory when conducting replicate analyses of a given sample, and the mean reduction of the bacterial content of a water sample as a function of the time elapsed after sampling, it was estimated that the faecal coliforms concentration of a water sample obtained 23 hours after sample collecting and the concentration obtained immediately after collection would not be statistically different with a 95% confidence level.

Figure 4 illustrates the faecal coliforms concentrations obtained by participating laboratories in the same series of replicate water samples. A comparison of the results shown in Figures 3 and 4 clearly points out the wide variations in relative precision achieved by different laboratories; while the concentrations obtained by some laboratories are well within the confidence interval of the reference laboratory, others are clearly outside that interval, indicating the presence of important sources of experimental error.

Without further statistical evaluation, a simple comparison between results in Figures 3 and 4 reveals the need for corrective action to bring the analytical precision within acceptable limits. In the intercalibration exercise under consideration, only 5 out of 11 laboratories obtained acceptable microbial results and, of those 5, only 2 laboratories obtained a precision comparable to that of the reference laboratory.

12.3.2 Assessment of water quality

One of the main objectives of a monitoring programme is to determine the conformity of the water sampling station to the criteria or standard under consideration. Consequently, the assessment made by different laboratories of the quality of a given sampling station can be used to verify the comparability of their analytical results. Instead of testing the precision of the basic microbiological results, this approach allows testing of the overall quality evaluation made by participating laboratories.

The experimental results obtained by the reference laboratory and the 5 participating laboratories previously selected were used to evaluate the mean microbial concentrations corresponding to the water sampling studied. The mean concentrations were derived using the lognormal probability model for interpreting microbial concentrations, as discussed in previous sections.

Figure 5 illustrates the mean bacterial concentrations obtained by each participating laboratory, as a function of the time elapsed after sample collection adopted by each laboratory. The experimental values obtained at the reference laboratory had been used to estimate the regression lines and the corresponding 95% confidence limits of the mean concentration values as a function of the time elapsed after sampling.

As Figure 5 indicates, the mean bacterial concentration obtained at the reference laboratory shows a slight reduction as the time elapsed after sample collection goes from 2 to 8 hours. The mean bacterial concentrations obtained by the other 5 laboratories fluctuate considerably around the values obtained by the reference laboratory. Although it has always been recommended to analyze the samples as soon

as possible after collection, transport and storage time may exceed 8 hours in some cases. These results illustrate the influence of storage time on the final assessment of the water quality made by different laboratories.

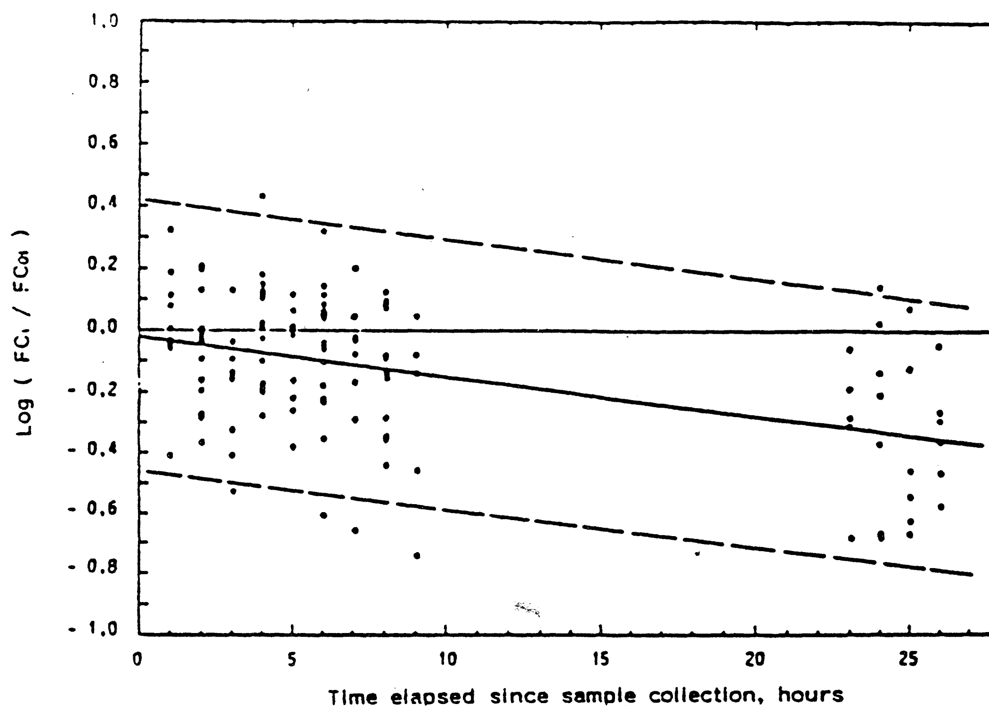

Figure 3. Faecal coliforms concentrations obtained by a reference laboratory as a function of the time elapsed since sample collection. Regression line and 95% confidence limits (Mujeriego, 1985).

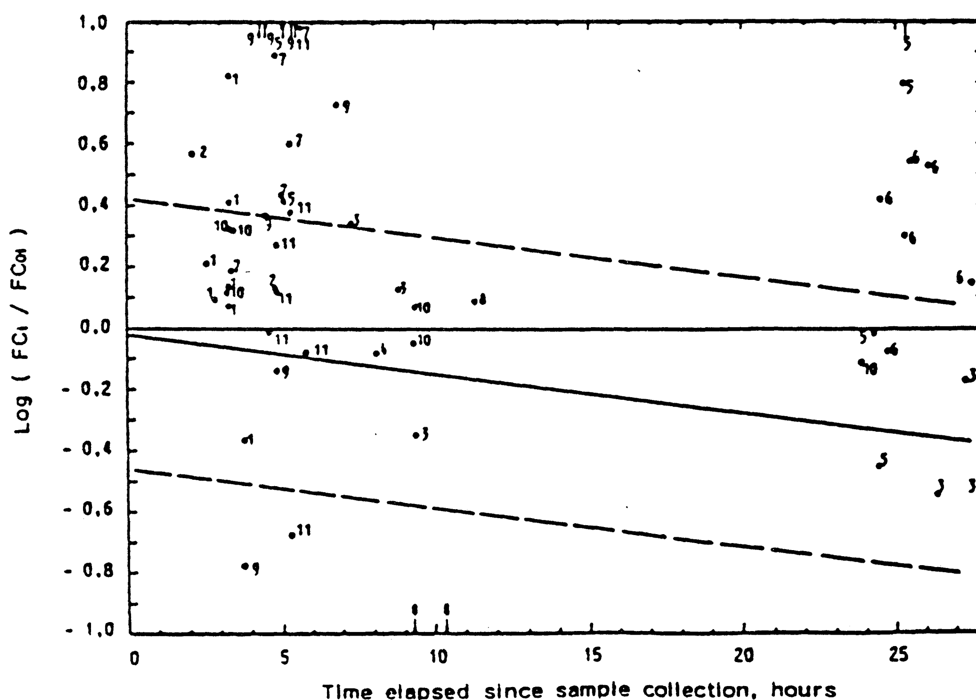

Figure 4. Faecal coliforms concentrations obtained by participating laboratories as a function of the time elapsed since sample collection. Regression line and 95% confidence limits of the reference laboratory results (Mujeriego, 1985).

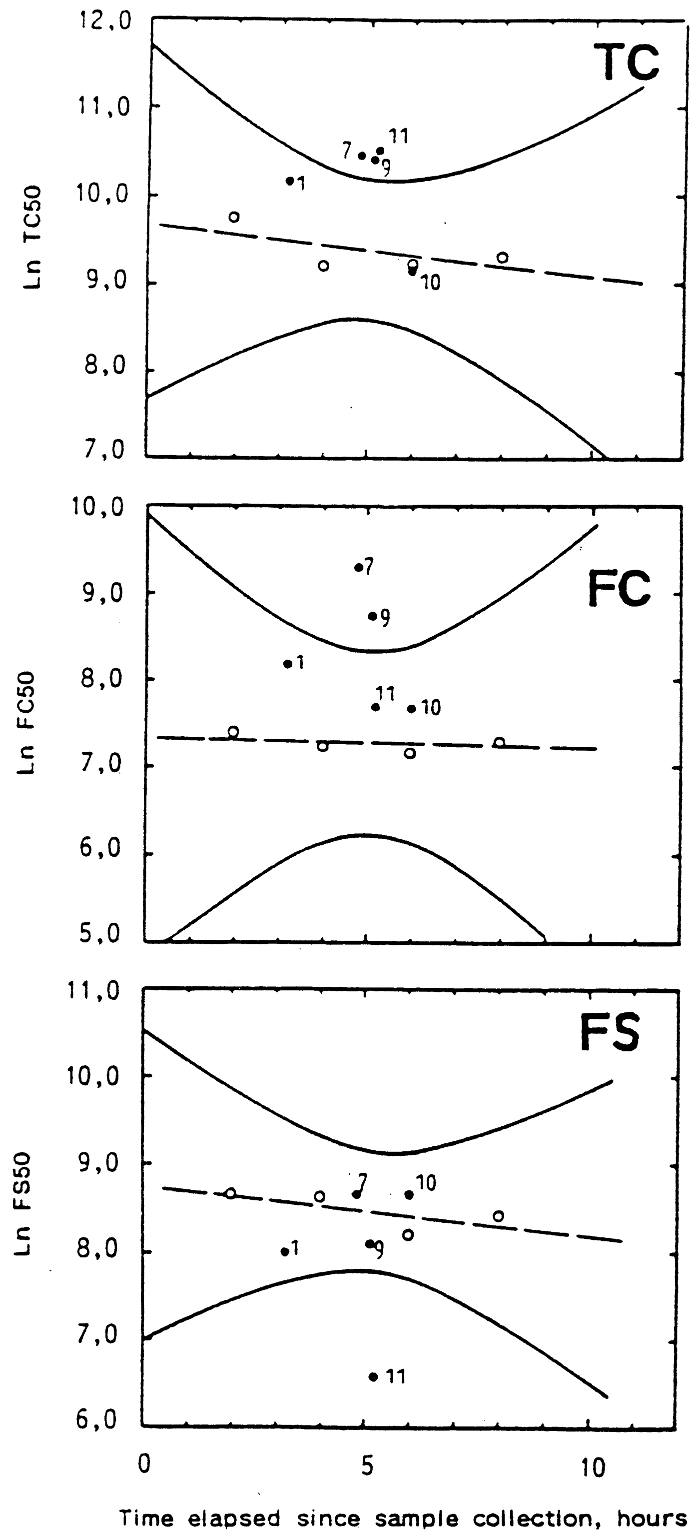

Figure 5. Mean bacterial concentrations obtained by the reference laboratory and by participating laboratories on chronological samples collected at a coastal water sampling station. Regression line and 95% confidence limits based on the results of the reference laboratory (Mujeriego, 1985).

Faecal streptococci examination appears to be the analytical method providing the most comparable results among laboratories. In particular, the discrepancy observed in laboratory number 11 was traced to the use of M-Enterococcus agar, instead of the KF-Streptococci agar used by other laboratories. Several Mediterranean laboratories (Yoshpe-Purer, 1989; Feliu, 1989) have studied the causes of the significantly larger counts obtained with the KF-Streptococci, and have shown the large number of false positive counts as the main reason for those discrepancies.

An overall assessment of the results shown in Figure 5 indicates that laboratory number 10 and, to a lesser extent, laboratory number 1 are those obtaining water quality evaluations comparable to that obtained by the reference laboratory. This approach provides therefore a useful tool for analytical quality control of microbiological laboratories.

In contrast to the approach discussed in the previous section, this procedure emphasizes a comparison between the overall evaluation of a water sampling station obtained by individual laboratories, instead of concentrating on caparisons between individual microbial examination of those same laboratories. Although the aim on both cases is to improve the quality of experimental results, one type of approach may be more suitable than the other, depending on the circumstances. Obviously, the statistical interpretation of the individual results has to be conducted according to identical protocols, as to prevent the introduction of an additional source of variation. The adoption of a reference method for interpretation of results appears thus as one essential element of any quality assurance programme.

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ANNEX 1

QUESTIONNAIRE FOR LABORATORY SURVEY

QUALITY CONTROL IN MICROBIOLOGICAL MONITORING OF COASTAL MARINE WATERS

The objective of this questionnaire is to provide useful advice when evaluating the need for and the degree of implementation of a quality assurance programme in microbiological laboratories engaged in monitoring coastal marine waters.

The questionnaire is intended for use by a survey expert during a personal visit to the laboratory facilities for direct observation and technical discussions with laboratory personnel on the procedures followed for microbiological analyses.

Futhermore, the person conducting the survey should gather detailed information on:

The organizational set up of the laboratory.

The scientific, technical and support personnel available.

The working space, the analytical equipment and the support equipment available at the laboratory.

The type of analyses normally conducted and the total amount of work carried out annually.

The quality assurance protocol available at the laboratory.

This information should be summarized and included as one chapter of the report to be prepared by the survey expert. This information will be very useful when interpreting the results of the quality assurance evaluation conducted according to the following questionnaire.

The following sections of the questionnaire summarize important aspects to be considered during the personal visit and technical discussion held with laboratory management and staff. The questionnaire is not intended to be completed by the laboratory personnel directly.

The material contained on Chapter 1 of the Guidelines for Quality Control in Microbiological Monitoring of Coastal Marine Water should help in establishing final recommendations on the quality assurance programme to be adopted in a specific laboratory.

SAMPLING PROCEDURES

1. Sampling Station Characteristics:

Water depth at sampling station
Type of base-line steady conducted to define sampling stations
Total number of sampling stations monitored
Total length of coastline monitored
Mean coastal length covered by one sampling station

Water sampling depth
Number of samples collected at each sampling station
- by categories (summer season/winter season, for example)
- average value annually

Time of day when samples are collected
Number of sampling station changed during last year

2. Sample Collection:

Sample volume normally collected
Sampling procedures: direct collection, rope or extension arm
Air space left in sample bottle when sampling
Systematic character of sample collection
Additional parameters and information collected during sampling, and sources of such information

3. Sample Identification:

Method of identification of sample bottles
Procedures for sample reception and registration at the laboratory

4. Sample Conservation:

Methods for sample conservation: temperature and light exposure
Conservation time before analysis

5. Sample Analysis

Category level of the laboratories actually analyzing the samples

LABORATORY EQUIPMENT

1. **Air Incubators:**

    Recording thermometers or direct reading thermometers
    Presence of 0.5 °C scale divisions
    Water reservoir for humidity control

2. **Elevated Temperature Thermometers:**

    Recording thermometers or direct reading thermometers
    Presence of 0.1 °C scale divisions
    Rust or salt deposits in water bath.

3. **Dry Heat Sterilization:**

    Equipment used: commercial type or home type ovens
    Sterilization temperature range used
    Availability of thermometers in the 160-180 °C range

4. **Autoclaves:**

    Equipment used: commercial autoclave or pressure cooker
    Availability of temperature control: position of thermometer
    Availability of pressure control: position of pressure valve
    State of internal walls, drains and rubber gaskets

5. **Thermometers:**

    Availability of certified thermometers for reference
    Procedures used for periodic calibration
    State of thermometers in use: mercury column integrity

6. **pH-meter:**

    Availability of a 0.1 pH unit precision instruments
    Procedures used for periodic calibration

7. **Balances:**

    Availability of a balance with sensitivity better than 2 g at 100 g load
    Availability of an analytical balance with sensitivity better than 1 mg at 10 g load

8. **Light Source:**

    Availability of a 10x to 15x magnification apparatus with a cold-white fluorescent lamp

9. Inoculating Tools:

Type of tools available: wire loops, hardwood sticks, Pasteur pipettes
Adequate size and characteristics of each type of tool
Autoclaving procedures used for each tool

10. Membrane Filtration Assemblies:

Types of assemblies available: glass, plastic, stainless-steel
Presence of dirt or deposits on funnel walls
Deterioration or deformation of funnels
Cleaning procedures used

11. Forceps

Forceps with smooth ends
Sterilization procedures used

LABORATORY UTENSILS

1. **Sampling Bottles:**

   Type of material: borosilicate glass, plastic, metal
   Type of closure: ground glass stoppers, plastic screw-cups
   Control of plastic cups toxicity
   Possible deformations due to autoclaving

2. **Pipettes:**

   Type of material: borosilicate glass, plastic
   Accuracy within 2.5%
   Sterilization containers: metal, wrapping paper

3. **Petri Dishes:**

   Size and type of material
   Disposable or reusable
   Sterilization containers: metal, wrapping paper
   Cleaning and sterilization procedures for reuse

4. **Culture Tubes and Bottles:**

   Size and type of material
   Methods for measuring dilution water volumes
   Closure for tubes and bottles: ground-glass stoppers, rubber or metal stoppers, plastic screw-caps

5. **Membrane Filters:**

   Brands, types and porosity in µm
   Cleaning and sterilization procedures for reuse

6. **Absorbent Pads:**

   Brands and types
   Alternative use of 1.5% agar media

CLEANING AND STERILIZATION

1. Cleaning of Glassware:

Cleaning methods: mechanical washing, hand washing
Type of detergent used: for laboratory use, home dish washing
Washing and rinsing procedures: use of distilled water
Visual inspection of dry glassware for sparkling clarity

2. Sterilization Procedures:

Media and reagents:

Autoclave for 10-12 minutes, and less than 15 minutes
Heating of MF culture media: in water bath for 5 minutes

Membrane filters and absorbent pads:

Presterilized or sterilized by autoclaving for 10 minutes
Precautions to prevent excessive exposure to heat

MF filtration equipment:

Autoclaving for 15 minutes, in wrapping paper
Availability of UV light for bench sterilization
Control of UV sterilization effectiveness
Dry heat sterilization of glass filter assemblies

Sample bottles:

Plastic bottles: autoclaving for 15 minutes
loosely closed caps for pressure equalization
Glass bottles: dry heat for 2 hours

Glassware items:

Sterilization by dry heat for 1 hour
Dry heat sterilization of hardwood sticks
Storage in metal foil/wrapped paper covers
Availability of metal containers for pipettes and glass Petri dishes
Sterilization in metal containers: dry heat for 2 hours

3. Dilution Water Blanks:

Autoclaving for 15 minutes
Careful timing of steam exhaust process
Provision of excess water volume to compensate for vapor losses

4. **Culture Dish Reuse:**

Procedures for plastic Petri dish reuse: repeated washing, ethylene oxide
Procedures for verifying the efficiency of sterilization procedure: standard plate count agar at 35 °C for 48 hours

5. **Flame Sterilization:**

Procedures for needles and wire loops: flame sterilization
Procedures for forceps and spatulas: alcohol flaming

6. **Laboratory Water Quality:**

Methods and equipments for producing distilled water: stainless-steel, glass systems
Presence of polyvinyl chloride tubing
Availability of commercial systems: cartridges replacement records and control of water quality
Conductivity measurements
Suitability test: frequency and results

7. **Dilution Water:**

Distilled water. 0.1% peptone water, or phosphate-buffered water (pH = 7.2)
Sampling processing time after dilution: less than 30 minutes
Control and storage of dilution water: turbidity, refrigeration

CULTURE MEDIA

1. Culture Media:

Brand and type: prepared dehydrated media, ampouled media, preweighed media
Broth dissolution: preheating in distilled water to 50 °C.
Agar dissolution: heating in water bath at 50 °C.
Media additives: rosolic acid only as inhibitory for noncoliform colonies

2. pH Measurements and Records:

Calibration with a pH = 7.0 standard buffer
Media pH deviations from tolerance limits: less than 0.5 pH units
pH measurement of sterilized culture media: at least one batch of each new media bottle

3. Media Storage:

Media stocked for less than a year and preferably for less than 6 months
Conservation of prepared media: protected form sunlight, contamination and excessive evaporation
Prepared media should be stocked less than 1 week

4. Media Quality Control:

Commercially prepared media: 2 years of shelf live
Periodic selectivity and recovery test

MULTIPLE TEST TUBE METHOD

1. **Total Coliforms:**

   Presumptive test reading: 24 hours at 35 °C
   culture media: lactose broth, lauryl tryptose broth
   Confirmed test reading: 48 hours at 35 °C
   culture media: brilliant green lactose broth (BGLB)
   Completed test

2. **Faecal Coliforms:**

   Incubation in EC broth for 24 hours at 44.5 ± 0.2 °C, subsequent to total coliforms presumptive test

3. **Faecal Streptococci:**

   Presumptive test: azide dextrose broth at 35 °C for 24 hours (or 48 hours)
   Confirmed test: Esculin-azide agar at 35 °C for 24 hours

4. **MPN Calculations:**

   Number of replicate tubes in a series and number of dilution series considered
   MPN tables used for calculations
   Procedures for determining the combination of positive tubes

Note: The corresponding Reference Methods for Marine Pollution Studies (WHO/UNEP) or Standard Methods (APHA, 1985) should be consulted for further details.

MEMBRANE FILTRATION METHOD

1. Laboratory and Routine Operational Practice:

Precision of water sample volumes
Vigorous shaking of sample bottles before pipetting
Dilution of water sample in filtration funnel before filtering
Repeated rinsing of the funnel after sample filtration
Dilution blank before filtration of water samples
Dilution blank in between filtration of water samples

2. Incubation and Counting of Colonies:

Total Coliforms:

Culture media used: M-Endo broth or agar (1.5%)
Incubation at 35 °C for 24 hours
Typical pink to dark red colour colonies with metallic surface sheen
Difficulties in counting small red colonies: practice and experience of personnel, together with results of the MPN confirmed test

Faecal Coliforms:

Water bath with precise temperature control: 44.5 ± 0.5 °C
Incubation for 24 hours
Culture media used: M-FC broth or agar (1.5%)
Typical blue colour colonies
Nonfaecal coliforms: gray to cream-coloured colonies

Faecal Streptococci:

Culture media used: M-Enterococcus agar, KF-Streptococcus agar
Incubation at 35 °C for 48 hours
Typical dark red to pink colonies

3. Replicate analyses:

Replicate analyses conducted periodically to verify analytical precision

INTERPRETATION OF RESULTS

1. Interpretation Methods:

Systematic and adequate application of interpretation methods: ranking method, lognornal probability method
Results available for each sampling station: range and average number of microbial concentrations
Variability of microbiological quality: typical values of the standard deviation
Adjustment to the lognormal probability model: possible causes or explanations
Experience gained and results obtained when interpreting experimental results

ANALYTICAL QUALITY CONTROL

1. Analytical Quality Control Programme:

Participation in intra-laboratory and intra-laboratory quality control programmes
Experience gained and results obtained from that participation

2. Intra-laboratory Control:

Methods used: control charts
Levels of precision of analytical analyses

3. Inter-laboratory control:

Sources and type of intercalibration samples
Methods used: lognormal probability method, regression analysis
Precision of individual microbiological concentrations
Precision of overall quality of sampling stations

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